EV889154183US

PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C07K 14/47, A61K 38/16, 38/17, 38/18 — A1

(11) International Publication Number: **WO 98/56819**

(43) International Publication Date: 17 December 1998 (17.12.98)

(21) International Application Number: PCT/US98/12345

(22) International Filing Date: 12 June 1998 (12.06.98)

(30) Priority Data:
60/049,578 13 June 1997 (13.06.97) US

(71) Applicant: THE BOARD OF TRUSTEES OF THE LELAND STANFORD JUNIOR UNIVERSITY [US/US]; Suite 350, 900 Welch Road, Palo Alto, CA 94304–1850 (US).

(72) Inventors: JONES, Patricia, P.; 639 Arastradero Road, Palo Alto, CA 94306 (US). CONBOY, Irina, B.; 350 Curtner, 12, Palo Alto, CA 94306 (US).

(74) Agent: SHERWOOD, Pamela, J.; Bozicevic & Reed LLP, Suite 200, 285 Hamilton Avenue, Palo Alto, CA 94301 (US).

(81) Designated States: AU, CA, JP, European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.

(54) Title: METHODS OF REGULATING T CELL AND MACROPHAGE CYTOKINE PRODUCTION, FUNCTION AND PATHOGENICITY

(57) Abstract

Methods and compositions are provided for the modulation of cytokine production by T helper cells and antigen–presenting cells. Compositions are provided that regulate the production of cytokines during the course of an immune response. The cytokine regulatory factor is produced by antigen presenting cells, and can act on uncommitted CD4 + cells to cause them to commit to Th1 cytokine phenotype, on mature, committed Th1 type T cells to decrease the synthesis of pro–inflammatory cytokines, and on macrophages to decrease the production of pro–inflammatory cytokines. The subject compositions can be used in diagnosis or therapy.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	**ES**	Spain	**LS**	Lesotho	**SI**	Slovenia
AM	Armenia	**FI**	Finland	**LT**	Lithuania	**SK**	Slovakia
AT	Austria	**FR**	France	**LU**	Luxembourg	**SN**	Senegal
AU	Australia	**GA**	Gabon	**LV**	Latvia	**SZ**	Swaziland
AZ	Azerbaijan	**GB**	United Kingdom	**MC**	Monaco	**TD**	Chad
BA	Bosnia and Herzegovina	**GE**	Georgia	**MD**	Republic of Moldova	**TG**	Togo
BB	Barbados	**GH**	Ghana	**MG**	Madagascar	**TJ**	Tajikistan
BE	Belgium	**GN**	Guinea	**MK**	The former Yugoslav Republic of Macedonia	**TM**	Turkmenistan
BF	Burkina Faso	**GR**	Greece			**TR**	Turkey
BG	Bulgaria	**HU**	Hungary	**ML**	Mali	**TT**	Trinidad and Tobago
BJ	Benin	**IE**	Ireland	**MN**	Mongolia	**UA**	Ukraine
BR	Brazil	**IL**	Israel	**MR**	Mauritania	**UG**	Uganda
BY	Belarus	**IS**	Iceland	**MW**	Malawi	**US**	United States of America
CA	Canada	**IT**	Italy	**MX**	Mexico	**UZ**	Uzbekistan
CF	Central African Republic	**JP**	Japan	**NE**	Niger	**VN**	Viet Nam
CG	Congo	**KE**	Kenya	**NL**	Netherlands	**YU**	Yugoslavia
CH	Switzerland	**KG**	Kyrgyzstan	**NO**	Norway	**ZW**	Zimbabwe
CI	Côte d'Ivoire	**KP**	Democratic People's Republic of Korea	**NZ**	New Zealand		
CM	Cameroon			**PL**	Poland		
CN	China	**KR**	Republic of Korea	**PT**	Portugal		
CU	Cuba	**KZ**	Kazakstan	**RO**	Romania		
CZ	Czech Republic	**LC**	Saint Lucia	**RU**	Russian Federation		
DE	Germany	**LI**	Liechtenstein	**SD**	Sudan		
DK	Denmark	**LK**	Sri Lanka	**SE**	Sweden		
EE	Estonia	**LR**	Liberia	**SG**	Singapore		

METHODS OF REGULATING T CELL AND MACROPHAGE CYTOKINE PRODUCTION, FUNCTION AND PATHOGENICITY

The development of this invention was supported at least in part under NIH grant RO1 AI9512, GM07276, HD07249, and by the Multiple Sclerosis Society grant PP0454. The government may have certain rights in this invention.

CROSS REFERENCE TO RELATED APPLICATIONS

This application claims priority to U.S. provisional patent application no. 60/049,578, filed June 13, 1997.

INTRODUCTION

Helper ($CD4^+$) T cells orchestrate the immune response of mammals through production of soluble factors that act on other immune system cells, including other T cells. Most mature $CD4^+$ T helper cells express one of two cytokine profiles: Th1 or Th2. Th1 cells secrete IL-2, IL-3, IFN-γ, TNF-β, GM-CSF and high levels of TNF-α. Th2 cells express IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL-13, GM-CSF and low levels of TNF-α. These cytokine profiles determine T cell regulatory and effector functions in immune responses. The Th1 subset promotes delayed-type hypersensitivity, cell-mediated immunity, and immunoglobulin class switching to IgG2a. The Th2 subset induces humoral immunity by activating B cells, promoting antibody production, and inducing class switching to IgG1 and IgE. Skewing the T cell responses toward Th1 is thought to result in susceptibility to autoimmune and inflammatory diseases; skewing toward Th2 cytokines promotes allergic reactions.

Several factors have been shown to influence commitment to Th1 or Th2 profiles. The best characterized regulators are cytokines. IL-12 and IFN-γ are positive Th1 and negative Th2 regulators. IL-12 promotes IFN-γ production, and IFN-γ provides positive feedback for IL-12. IL-4 and IL-10 appear to be required for the establishment of the Th2 cytokine profile and to down-regulate Th1 cytokine production; the effects of IL-4 have been demonstrated to be dominant over those of IL-12. IL-13 was shown to inhibit expression of inflammatory cytokines, including IL-12 and TNF-α by LPS-induced monocytes, in a way similar to IL-4.

Signals through the T cell receptor (TCR) and co-stimulatory molecules have also been shown to influence Th commitment. Th1 development was found to be associated with high affinity binding of a peptide antigen to MHC class II and strong signaling through the TCR, whereas lower-affinity antigen-MHC II interactions and weaker signaling through TCR were reported to result in Th2 cytokine responses (Constant *et al.* (1995) *J. Exp. Med.* **182**:1501-1596).

Several cell surface molecules expressed by antigen presenting cells (APC) and T cells have been suggested to influence commitment to Th1 or Th2 response, including CD40-CD40 ligand interactions and B7.1 *vs.* B7.2 signaling. At the cellular level, at least in some cases, macrophages and dendritic cells appear to promote the Th1 response, whereas B cells upregulate Th2 cytokines. However, neither the B7.1/B7.2 effects nor the effects of different APC types in supporting Th1 or Th2 are absolute.

The role of T cells in autoimmune disease is of great interest. There are a number of mechanisms that cooperate to produce and maintain T-cell tolerance. During thymic education, T cells with high affinity for self antigens are clonally deleted. However, to ensure that a wide repertoire of T cells is available in the periphery, the threshold for clonal deletion may be set low enough so that T cells whose antigen receptors have sub-threshold affinity for self antigens mature and migrate to the periphery. T cells that recognize self antigen-derived peptides not expressed or presented in the thymus will also fail to be deleted. Self-reactive T cells that are not deleted in the thymus may be tolerized through other mechanisms, including an alteration of signaling pathways to produce clonal anergy, and lowering the avidity of the TCR for its ligand by downregulating coreceptor and accessory molecules. Active suppression of T-cell responses may also be in effect. It has been suggested by Liu *et al.* (1995) Immunity **3**:407-415 that low avidity recognition of self-antigen by T cells permits escape from central tolerance. An encephalitogenic epitope of myelin basic protein has been shown to have low affinity for the presenting MHC molecule. The avidity of T cell recognition in the thymus may be compromised, enabling autoreactive T cells to escape self-tolerance. The specific effects of T cell reactivity toward autoantigens is of great interest for the diagnosis and treatment of autoimmune disease. Methods of altering the cytokine profile of autoreactive T cells would be beneficial in the treatment of autoimmune disease.

Relevant Literature

The nucleotide and amino acid sequence of the protein previously identified as cyclophilin C-associated protein (CyCAP) is available at Genbank, accession number L16894. For convenience, the complete coding sequence of the mouse CyCAP and its encoded polypeptide are provided as SEQ ID NO:1 and SEQ ID NO:2. The protein is also known as 90 K and murine adherent macrophage protein. The human homolog has been previously described as Mac-2-binding protein, which is available at Genbank, accession number 1082577. The nucleotide and amino acid sequences are provided herein as SEQ ID NO:3 and SEQ ID NO:4, respectively. For convenience, the polypeptides are referred to herein as cytokine regulatory factor (CYTRF) generically, and may be further designated as mouse, human, *etc.*

The cloning and characterization of CyCAP is described in Friedman *et al.* (1993) P.N.A.S. **90**:6815-6819. The human homolog was independently cloned by a number of

researchers, and is described in Rosenberg *et al.* (1991) J. Biol. Chem. **266**:18731-18736; Iacobelli et al. (1993) FEBS Lett. 319:59-65; Koths *et al.* (1993) J. Biol. Chem. **268**:14245-14249; Inohara *et al.* (1994) Biochem Biophys. Res. Comm. **201**:1366-1375; and Ullrich *et al.* (1994) J. Biol. Chem. **269**:18401-18407.

Other biological activities of CyCAP (90K protein, Mac-2-binding protein, murine adherent macrophage protein) are described in: Chicheportiche and Vassalli (1994) J. Biol. Chem. **269**:5512-5517; Natoli *et al.* (1993) J. Acquired Imm. Deficiency Syndrome **6**:370-375; Brakebusch *et al.* (1997) J. Biol. Chem. **272**:3674-3682; Pearson (1996) Curr. Op. Immunol. **8**:20-28; and Ma *et al.* (1996) J. Exp. Med. **183**:147-157.

SUMMARY OF THE INVENTION

Methods are provided for the modulation of cytokine production by T helper cells. A pharmaceutical composition comprising CYTRF as an active agent is administered *in vitro* or *in vivo*, and can act on mature, committed Th1 type T cells to decrease the synthesis of pro-inflammatory cytokines, or to skew the commitment of precursor T helper cells to Th1 or a Th1 cytokine profile. It is particularly effective for T cells having weak response to the stimulating antigen. The inflammatory response of T cells associated with disease conditions is suppressed by the delivery of CYTRF. The administered CYTRF may be a naturally occurring form of the protein, or a synthetic variant derived therefrom. CYTRF signaling and related pathways are also useful for modeling and screening novel pharmacological agents.

BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1A-1F. Cytokine levels of MBP-specific T cell lines stimulated overnight with syngeneic APC.

Figures 2A-2E. Cytokines produced by T cell lines (TCL 3) from B10.A and B10.BR mice are influenced by the source of APC (B10.A, B10.BR, or F1) used for *in vitro* re-stimulations.

Figures 3A-3C. Down-regulation of B10.BR TCL IFN-γ and TNF-α production by B10.A APC or by 2-hour supernatant from activated B10.A APC.

Figures 4A-4C. Supernatant from B10.A T cells activated with B10.A APC for 2 hours down-regulates IFN-γ and TNF-α production by B10.BR Th1 cells when A^k-expressing L cell fibroblasts are used as APC.

Figures 5A-5C. Down-regulation of IFN-γ and TNF-α levels is not affected when 2-hour B10.A culture supernatant is depleted of known Th1/Th2 cytokine regulators.

Figures 6A-6B. Modulation of down-regulatory activity by addition or neutralization of known cytokine regulators during re-stimulation of B10.BR T cells with either B10.BR or B10.A splenic APC.

Figures 7A-7B. Down-regulatory effects of B10.A splenic APC on IFN-γ and TNF-α production by a B10.BR-derived KLH-specific, E^k-restricted T cell clone.

Figure 8. Down-regulation of TNF-a production by purified populations of B10.A splenocytes.

Figures 9A and 9B. Biochemical properties of the Th1 down-regulatory factor.

Figures 10A-10B. Depletion of the B10.A-derived down-regulatory activity with anti-CyCAP antibodies or with the Cyp C-GST fusion protein.

Figures 11A-11D. Purified CyCAP down-regulates TNF-α and IFN-γ production, but not the T cell proliferation.

Figures 12A-12B. Proliferative responses to MBP Ac 1-16 and Hb 64-76 peptides.

Figures 13A-13B. The B10.A-derived Th1 down-regulatory activity depends on the strength of T cell activation.

Figures 14A-14B. Signaling by a partial agonist is dominant over strong agonist in permitting down-regulation of TNF-α and IFN-γ.

Figures 15A-15C. Effects of CyCAP on Th1 cytokine production in response to strong and to partial agonists.

Figures 16A-16B. IL-12 production by B10.A, B10.BR, (B10.A x B10.BR)F1, CyCAP $^{+/+}$, and CyCAP $^{-/-}$ macrophages.

DESCRIPTION OF THE SPECIFIC EMBODIMENTS

Methods and compositions are provided for directing the Th1/Th2 phenotype of T cell subsets, using the protein CYTRF. The protein is effective at suppressing Th1 type responses, particularly in response to weak antigens. CYTRF also inhibits the synthesis of IL-12 by antigen presenting cells, *e.g.* macrophages, dendritic cells, *etc.* CYTRF may be administered alone or in combination with other active agents to a patient suffering from pro-inflammatory conditions, *e.g.* T cell mediated autoimmune diseases. Methods of blocking of CYTRF may also be used, where a pro-inflammatory response is desired.

The subject methods utilize a novel activity of a previously known polypeptide, herein referred to as CYTRF. The protein sequences are provided in the SEQLIST, and are publically known, as described above. It has been found that CYTRF suppresses the synthesis of pro-inflammatory cytokines, *e.g.* tumor necrosis factor-α (TNF-α) and γ-interferon (γ-IFN) The synthesis of interleukin-2 (IL-2), which is involved in all T cell mediated responses, is not affected by the subject methods.

Cytokines produced by Th1 type T cells are associated with inflammation, where capillaries are dilated, fluids accumulate, and migration of phagocytic leukocytes, such as granulocytes and monocytes, to the site of injury or lesion is seen. Inflammation is important in defending a host against a variety of infections, but can also have undesirable consequences in inflammatory disorders. CYTRF is useful in modulating the T cell response by down-regulating expression of pro-inflammatory cytokines, and in studying the substrates, receptors and pathways of this important biological process.

Conditions characterized by the undesirable release of pro-inflammatory cytokines include autoimmune diseases, inflammation caused by bacterial, viral and parasitic infection, including response to vaccination, skin sensitivity, e.g. response to plant toxins or irritants, local inflammation in response to trauma, graft rejection, graft *v.* host disease, and the like. Administration of CYTRF is used to decrease the release of γ-IFN and TNF-α by activated T cells, and to shift the initial response to a Th2 type. Administration may be local or systemic.

Blocking the action of CYTRF by the adminstration of agents that interfere with its biological activity or receptor binding is useful in shifting the initial response to Th1 type. This decreases undesirable allergic responses, as may be associated with asthma, *etc.*

Of particular interest are T cell responses to weak antigens, e.g. the response of post-thymic T cells to autoantigens. As used herein, weak antigens are those peptides that either bind weakly to MHC proteins on antigen-presenting cells, or form MHC:peptide complexes that are recognized weakly by T cell receptors (TCR). A weak response may be experimentally characterized. Methods are known in the art for measuring the response of a T cell to an antigen, e.g. by assaying 3H-thymidine incorporation into the DNA of responding T cells, or production of specific cytokines by responding cells, such as IL-2, IFN-γ, *etc.*, where the proliferation of a responding cell to an antigen is measured. A negative control of no antigen is taken to be a null response. The maximal response may be elicited, for example, by stimulating T cells with syngeneic antigen presenting cells and varying concentrations of protein or peptide antigen. The spectrum of responses ranging from negative to maximal may be arranged according to strength of response (e.g. proliferation) over 3 logs of arbitrary units. The antigen providing for the lowest log of response are negative, the next log are weak antigens and the maximal response are the strongest antigens. Generally, a weak antigen will provide a response that may require antigen concentrations of at least about 1 μM, or higher. The strong or weak nature of the antigen when bound as a peptide to an MHC protein can also be assessed by the induced changes in the T cell, e.g. phosphorylation of TCR/CD3-associated polypeptides, or down-regulation of TCR expression.

Characterization of a peptide may also be measured by the affinity of a peptide for the presenting MHC, or by the avidity of the responding T cells for the peptide/MHC complex, using methods known in the art. For example, Alam *et al.* (1996) Nature **381**:616-620 describe the use

of surface plasmon resonance to measure the kinetics of TCR interactions ligands. Jorgensen *et al.* (1992) Annu Rev Immunol **10**:835-873 review the ternary complex of the T cell receptor (TCR), antigenic peptides, and molecules of the major histocompatibility complex (MHC). A measure of the affinity of TCR for peptide/MHC complexes comes from competition experiments using soluble MHC complexed with specific peptides.

Methods of Use

Formulations of CYTRF are administered to a host suffering from a pro-inflammatory immune disorder. Macrophages are major producers of TNF-α, and IL-12, which is a major inducer of a Th1 type response. Synthesis of these cytokines by macrophages is inhibited by the administration of CYTRF. Administration may be topical, localized or systemic, depending on the specific condition. Generally the dose will be sufficient to decrease the synthesis of pro-inflammatory cytokines by at least about 50%, and may be higher, at least about 90%, or as much as 99% or higher. The compounds of the present invention are administered at a dosage that reduces the cytokine synthesis while minimizing any side-effects. It is contemplated that the composition will be obtained and used under the guidance of a physician for *in vivo* use.

Autoimmune diseases of interest are associated with T cell-mediated or macrophage-mediated tissue destruction. Included are multiple sclerosis (MS), rheumatoid arthritis (RA), reactive arthritis, psoriasis, pemphigus vulgaris, Sjogren's disease, thyroid disease, Hashimoto's thyroiditis, myasthenia gravis, insulin dependent diabetes mellitus (IDDM), ulcerative colitis/Crohn's disease, as well as many others. Treatment of primates, more particularly humans is of interest, but other mammals may also benefit from treatment, particularly domestic animals such as equine, bovine, ovine, feline, canine, murine, lagomorpha, and the like.

The subject therapy will desirably be administered during the presymptomatic or preclinical stage of the disease, and in some cases during the symptomatic stage of the disease. Early treatment is preferable, in order to prevent the loss of function associated with autoimmune tissue damage. The presymptomatic, or preclinical stage will be defined as that period not later than when there is T cell involvement at the site of disease, *e.g.* islets of Langerhans, synovial tissue, thyroid gland, *etc.*, but the loss of function is not yet severe enough to produce the clinical symptoms indicative of overt disease. T cell involvement may be evidenced by the presence of elevated numbers of T cells at the site of disease, the presence of T cells specific for autoantigens, the release of cytokines, perforins and granzymes at the site of disease, response to immunosuppressive therapy, *etc.*

Degenerative joint diseases may be inflammatory, as with seronegative spondylarthropathies, *e.g.* ankylosing spondylitis and reactive arthritis; rheumatoid arthritis; gout; and systemic lupus erythematosus. The degenerative joint diseases have the common feature in

that the cartilage of the joint is eroded, eventually exposing the bone surface. Destruction of cartilage begins with the degradation of proteoglycan, mediated by enzymes such as stromelysin and collagenase, resulting in the loss of the ability to resist compressive stress. Alterations in the expression of adhesion molecules, such as CD44 (Swissprot P22511), ICAM-1 (Swissprot P05362), and extracellular matrix protein, such as fibronectin and tenascin, follow. Eventually fibrous collagens are attacked by metalloproteases, and when the collagenous microskeleton is lost, repair by regeneration is impossible. There is significant immunological activity within the synovium during the course of inflammatory arthritis. While treatment during early stages is desirable, the adverse symptoms of the disease may be at least partially alleviated by treatment during later stages. Clinical indices for the severity of arthritis include pain, swelling, fatigue and morning stiffness, and may be quantitatively monitored by Pannus criteria. Disease progression in animal models may be followed by measurement of affected joint inflammation. Therapy for inflammatory arthritis may combine the subject treatment with conventional NSAID treatment.

A quantitative increase in myelin-autoreactive T cells with the capacity to secrete IFN-gamma is associated with the pathogenesis of MS, suggesting that autoimmune inducer/helper T lymphocytes in the peripheral blood of MS patients may initiate and/or regulate the demyelination process in patients with MS. The overt disease is associated with muscle weakness, loss of abdominal reflexes, visual defects and paresthesias. During the presymptomatic period there is infiltration of leukocytes into the cerebrospinal fluid, inflammation and demyelination. Family histories and the presence of the HLA haplotype DRB1*1501, DQA1*0102, DQB1*0602 are indicative of a susceptibility to the disease. Markers that may be monitored for disease progression are the presence of antibodies in the cerebrospinal fluid, "evoked potentials" seen by electroencephalography in the visual cortex and brainstem, and the presence of spinal cord defects by MRI or computerized tomography. Treatment during the early stages of the disease will slow down or arrest the further loss of neural function.

Human IDDM is a cell-mediated autoimmune disorder leading to destruction of insulin-secreting b cells and overt hyperglycemia. T lymphocytes invade the islets of Langerhans, and specifically destroy insulin-producing β-cells. The depletion of β-cells results in an inability to regulate levels of glucose in the blood. Overt diabetes occurs when the level of glucose in the blood rises above a specific level, usually about 250 mg/dl. In humans a long presymptomatic period precedes the onset of diabetes. During this period there is a gradual loss of pancreatic β-cell function. The disease progression may be monitored in individuals diagnosed by family history and genetic analysis as being susceptible. The most important genetic effect is seen with genes of the major histocompatibility locus (*IDDM1*), although other loci, including the insulin gene region (*IDDM2*) also show linkage to the disease (see Davies *et al, supra* and Kennedy *et al.* (1995) Nature Genetics 9:293–298). Markers that may be evaluated during the presymptomatic stage are the presence of insulitis in the pancreas, the level and frequency of islet cell antibodies,

islet cell surface antibodies; aberrant expression of Class II MHC molecules on pancreatic b cells, glucose concentration in the blood, and the plasma concentration of insulin. An increase in the number of T lymphocytes in the pancreas, islet cell antibodies and blood glucose is indicative of the disease, as is a decrease in insulin concentration. After the onset of overt diabetes, patients with residual β-cell function, evidenced by the plasma persistence of insulin C-peptide, may also benefit from administration of the subject polysaccharides in order to prevent further loss of function.

Inflammatory diseases caused by bacterial and viral infection include viral meningitis and bacterial meningitis, herpes encephalitis and viral meningoencephalitis, viral hepatitis, *e.g.* Hepatitis A, B, C, D, *etc.* Diseases of interest also include inflammatory response to vaccination, particularly rabies vaccine, varicella zoster vaccine, measles vaccine, *etc.*

CYTRF Compounds

The cytokine regulatory factor, CYTRF is characterized as an acidic glycoprotein having a molecular weight of approximately 100 Kdal or more, determined by size exclusion centrifugation and chromatography, and having the properties of binding to anti-CyCAP antibodies and cyclophilin C. When added to Th1 committed T cells, the factor down-regulates expression of the pro-inflammatory cytokines IFN-γ and TNF-α. CYTRF also inhibits production of the pro-Th1 cytokine IL-12 by macrophages. When the factor is present in stimulated cultures of Th0 cells, it shifts the cytokine profile of the stimulated T cells to a Th2 type. In mice, CYTRF activity is genetically upregulated by a non-*H2* linked locus derived from A strain, *e.g.* B10.A, A/WySn, and absent in mice having a C57BL background, *e.g.* B10.BR.

For use in the subject methods, any of the native CYTRF forms, modifications thereof, or a combination of one or more forms may be used. The CYTRF sequence may be from any mammalian or avian species, *e.g.* primate *sp.*, particularly humans; rodents, including mice, rats and hamsters; rabbits; equines, bovines, canines, felines; *etc.* Of particular interest are the human proteins. Generally, for *in vivo* use the CYTRF sequence will have the same species of origin as the animal host. For *in vitro* use, any convenient species having high activity against the microbe being treated may be used.

The nucleic acid sequences encoding the above human CYTRF polypeptide may be accessed from public databases as previously cited. Identification of non-human CYTRFs is accomplished by conventional screening methods of DNA libraries or biological samples for DNA sequences having a high degree of similarity to known CYTRF sequences.

The sequence of the CYTRF polypeptide may be altered in various ways known in the art to generate targeted changes in sequence. The polypeptide will usually be substantially similar to

the sequences provided herein, *i.e.* will differ by at least one amino acid, and may differ by at least two but not more than about ten amino acids. The sequence changes may be substitutions, insertions or deletions. Scanning mutations that systematically introduce alanine, or other residues, may be used to determine key amino acids. Deletions may further include larger changes, such as deletions of a domain or exon. Other modifications of interest include epitope tagging, *e.g.* with the FLAG system, HA, *etc.* Such alterations may be used to alter properties of the protein, by affecting the stability, specificity, *etc.*

Techniques for *in vitro* mutagenesis of cloned genes are known. Examples of protocols for scanning mutations may be found in Gustin *et al.*, *Biotechniques* 14:22 (1993); Barany, *Gene* 37:111-23 (1985); Colicelli *et al.*, *Mol Gen Genet* 199:537-9 (1985); and Prentki *et al.*, *Gene* 29:303-13 (1984). Methods for site specific mutagenesis can be found in Sambrook *et al.*, *Molecular Cloning: A Laboratory Manual*, CSH Press 1989, pp. 15.3-15.108; Weiner *et al.*, *Gene* 126:35-41 (1993); Sayers *et al.*, *Biotechniques* 13:592-6 (1992); Jones and Winistorfer, *Biotechniques* 12:528-30 (1992); Barton *et al.*, *Nucleic Acids Res* 18:7349-55 (1990); Marotti and Tomich, *Gene Anal Tech* 6:67-70 (1989); and *Zhu Anal Biochem* 177:120-4 (1989).

The protein may be joined to a wide variety of other oligopeptides or proteins for a variety of purposes. By providing for expression of the subject peptides, various post-expression modifications may be achieved. For example, by employing the appropriate coding sequences, one may provide farnesylation or prenylation. In this situation, the subject peptide will be bound to a lipid group at a terminus, so as to be able to be bound to a lipid membrane, such as a liposome.

The subject peptides may be PEGylated, where the polyethyleneoxy group provides for enhanced lifetime in the blood stream. The subject peptides may also be combined with other proteins, such as the Fc of an IgG isotype, which may be complement binding, with a toxin, such as ricin, abrin, diphtheria toxin, or the like, or with specific binding agents that allow targeting to specific moieties on a target cell.

Where targeting is desired, the active domain of CYTRF may be produced as a fusion protein with an antibody that is specific for a target cell of interest, thereby providing for an antimicrobial antibody composition. The antibody may be produced as a single chain, instead of the normal multimeric structure. Single chain antibodies are described in Jost *et al.* (1994) J.B.C. **269**:26267–73, and others. DNA sequences encoding the variable region of the heavy chain and the variable region of the light chain are ligated to a spacer encoding at least about 4 amino acids of small neutral amino acids, including glycine and/or serine. The protein encoded by this fusion allows assembly of a functional variable region that retains the specificity and affinity of the original antibody.

Formulations

The compounds of this invention can be incorporated into a variety of formulations for therapeutic administration. More particularly, the compounds of the present invention can be formulated into pharmaceutical compositions by combination with appropriate, pharmaceutically acceptable carriers or diluents, and may be formulated into preparations in solid, semi-solid, liquid or gaseous forms, such as tablets, capsules, powders, granules, ointments, solutions, suppositories, injections, inhalants, gels, microspheres, and aerosols. As such, administration of the compounds can be achieved in various ways, including oral, buccal, rectal, parenteral, intraperitoneal, intradermal, transdermal, intracheal, *etc.*, administration. The CYTRF may be systemic after administration or may be localized by the use of an implant that acts to retain the active dose at the site of implantation.

The compounds of the present invention can be administered alone, in combination with each other, or they can be used in combination with other known compounds (*e.g.*, perforin, antibiotics, *etc.*) In pharmaceutical dosage forms, the compounds may be administered in the form of their pharmaceutically acceptable salts, or they may also be used alone or in appropriate association, as well as in combination with other pharmaceutically active compounds. The following methods and excipients are merely exemplary and are in no way limiting.

For oral preparations, the compounds can be used alone or in combination with appropriate additives to make tablets, powders, granules or capsules, for example, with conventional additives, such as lactose, mannitol, corn starch or potato starch; with binders, such as crystalline cellulose, cellulose derivatives, acacia, corn starch or gelatins; with disintegrators, such as corn starch, potato starch or sodium carboxymethylcellulose; with lubricants, such as talc or magnesium stearate; and if desired, with diluents, buffering agents, moistening agents, preservatives and flavoring agents.

The compounds can be formulated into preparations for injections by dissolving, suspending or emulsifying them in an aqueous or nonaqueous solvent, such as vegetable or other similar oils, synthetic aliphatic acid glycerides, esters of higher aliphatic acids or propylene glycol; and if desired, with conventional additives such as solubilizers, isotonic agents, suspending agents, emulsifying agents, stabilizers and preservatives.

The compounds can be utilized in aerosol formulation to be administered via inhalation. The compounds of the present invention can be formulated into pressurized acceptable propellants such as dichlorodifluoromethane, propane, nitrogen and the like.

Furthermore, the compounds can be made into suppositories by mixing with a variety of bases such as emulsifying bases or water-soluble bases. The compounds of the present invention can be administered rectally via a suppository. The suppository can include vehicles such as

cocoa butter, carbowaxes and polyethylene glycols, which melt at body temperature, yet are solidified at room temperature.

Unit dosage forms for oral or rectal administration such as syrups, elixirs, and suspensions may be provided wherein each dosage unit, for example, teaspoonful, tablespoonful, tablet or suppository, contains a predetermined amount of the composition containing one or more compounds of the present invention. Similarly, unit dosage forms for injection or intravenous administration may comprise the compound of the present invention in a composition as a solution in sterile water, normal saline or another pharmaceutically acceptable carrier.

Implants for sustained release formulations are well-known in the art. Implants are formulated as microspheres, slabs, *etc.* with biodegradable or non-biodegradable polymers. For example, polymers of lactic acid and/or glycolic acid form an erodible polymer that is well-tolerated by the host. The implant containing CYTRF is placed in proximity to the site of infection or inflammation, so that the local concentration of active agent is increased relative to the rest of the body.

The term "unit dosage form," as used herein, refers to physically discrete units suitable as unitary dosages for human and animal subjects, each unit containing a predetermined quantity of compounds of the present invention calculated in an amount sufficient to produce the desired effect in association with a pharmaceutically acceptable diluent, carrier or vehicle. The specifications for the novel unit dosage forms of the present invention depend on the particular compound employed and the effect to be achieved, and the pharmacodynamics associated with each compound in the host.

The pharmaceutically acceptable excipients, such as vehicles, adjuvants, carriers or diluents, are readily available to the public. Moreover, pharmaceutically acceptable auxiliary substances, such as pH adjusting and buffering agents, tonicity adjusting agents, stabilizers, wetting agents and the like, are readily available to the public.

Typical dosages for systemic administration range from 0.1μg to 100 milligrams per kg weight of subject per administration. A typical dosage may be one tablet taken from two to six times daily, or one time-release capsule or tablet taken once a day and containing a proportionally higher content of active ingredient. The time-release effect may be obtained by capsule materials that dissolve at different pH values, by capsules that release slowly by osmotic pressure, or by any other known means of controlled release.

Those of skill will readily appreciate that dose levels can vary as a function of the specific compound, the severity of the symptoms and the susceptibility of the subject to side effects. Some of the specific compounds are more potent than others. Preferred dosages for a given compound are readily determinable by those of skill in the art by a variety of means. A preferred means is to measure the physiological potency of a given compound.

The use of liposomes as a delivery vehicle is one method of interest. The liposomes fuse with the cells of the target site and deliver the contents of the lumen intracellularly. The liposomes are maintained in contact with the cells for sufficient time for fusion, using various means to maintain contact, such as isolation, binding agents, and the like. In one aspect of the invention, liposomes are designed to be aerosolized for pulmonary administration. Liposomes may be prepared with purified proteins or peptides that mediate fusion of membranes, such as Sendai virus or influenza virus, *etc.* The lipids may be any useful combination of known liposome forming lipids, including cationic lipids, such as phosphatidylcholine. The remaining lipid will normally be neutral lipids, such as cholesterol, phosphatidyl serine, phosphatidyl glycerol, and the like.

For preparing the liposomes, the procedure described by Kato *et al.* (1991) J. Biol. Chem. **266**:3361 may be used. Briefly, the lipids and lumen composition containing the nucleic acids are combined in an appropriate aqueous medium, conveniently a saline medium where the total solids will be in the range of about 1-10 weight percent. After intense agitation for short periods of time, from about 5-60 sec., the tube is placed in a warm water bath, from about 25-40° C and this cycle repeated from about 5-10 times. The composition is then sonicated for a convenient period of time, generally from about 1-10 sec. and may be further agitated by vortexing. The volume is then expanded by adding aqueous medium, generally increasing the volume by about from 1-2 fold, followed by shaking and cooling. This method allows for the incorporation into the lumen of high molecular weight molecules.

As an alternative method of delivery, the CYTRF genes are used for modulating expression *in vitro* and *in vivo*. Vectors useful for introduction of the gene include plasmids and viral vectors. Of particular interest are retroviral-based vectors, *e.g.* Moloney murine leukemia virus and modified human immunodeficiency virus; adenovirus vectors, *etc.* that are maintained transiently or stably in mammalian cells. A wide variety of vectors can be employed for transfection and/or integration of the gene into the genome of the cells. Alternatively, micro-injection may be employed, fusion, or the like for introduction of genes into a suitable host cell. See, for example, Dhawan *et al.* (1991) Science **254**:1509-1512 and Smith *et al.* (1990) Molecular and Cellular Biology 3268-3271.

The expression vector will have a transcriptional initiation region oriented to produce functional mRNA. The native transcriptional initiation region or an exogenous transcriptional initiation region may be employed. The promoter may be introduced by recombinant methods *in vitro*, or as the result of homologous integration of the sequence into a chromosome. Many strong promoters are known in the art, including the β-actin promoter, SV40 early and late promoters, human cytomegalovirus promoter, retroviral LTRs, methallothionein responsive element (MRE), tetracycline-inducible promoter constructs, *etc.*

Expression vectors generally have convenient restriction sites located near the promoter sequence to provide for the insertion of nucleic acid sequences. Transcription cassettes may be prepared comprising a transcription initiation region, the target gene or fragment thereof, and a transcriptional termination region. The transcription cassettes may be introduced into a variety of vectors, *e.g.* plasmid; retrovirus, *e.g.* lentivirus; adenovirus; and the like, where the vectors are able to transiently or stably be maintained in the cells, usually for a period of at least about one day, more usually for a period of at least about several days to several weeks.

CYTRF Blocking Agents

The CYTRF polypeptides are used for the production of antibodies, where short fragments provide for antibodies specific for the particular polypeptide, and larger fragments or the entire protein allow for the production of antibodies over the surface of the polypeptide. Antibodies may be raised to the wild-type or variant forms of CYTRF. Antibodies may be raised to isolated peptides corresponding to these domains, or to the native protein, *e.g.* by immunization with cells expressing CYTRF, immunization with liposomes having CYTRF inserted in the membrane, *etc.*

Antibodies are prepared in accordance with conventional ways, where the expressed polypeptide or protein is used as an immunogen, by itself or conjugated to known immunogenic carriers, *e.g.* KLH, pre-S HBsAg, other viral or eukaryotic proteins, or the like. Various adjuvants may be employed, with a series of injections, as appropriate. For monoclonal antibodies, after one or more booster injections, the spleen is isolated, the lymphocytes immortalized by cell fusion, and then screened for high affinity antibody binding. The immortalized cells, *i.e.* hybridomas, producing the desired antibodies may then be expanded. For further description, see Monoclonal Antibodies: A Laboratory Manual, Harlow and Lane eds., Cold Spring Harbor Laboratories, Cold Spring Harbor, New York, 1988. If desired, the mRNA encoding the heavy and light chains may be isolated and mutagenized by cloning in *E. coli*, and the heavy and light chains mixed to further enhance the affinity of the antibody.

Antisense molecules are used to down-regulate expression of CYTRF in cells. The anti-sense reagent may be antisense oligonucleotides (ODN), particularly synthetic ODN having chemical modifications from native nucleic acids, or nucleic acid constructs that express such anti-sense molecules as RNA. The antisense sequence is complementary to the mRNA of the targeted gene, and inhibits expression of the targeted gene products. Antisense molecules inhibit gene expression through various mechanisms, *e.g.* by reducing the amount of mRNA available for translation, through activation of RNAse H, or steric hindrance. One or a combination of antisense molecules may be administered, where a combination may comprise multiple different sequences.

Drug Screening

The CYTRF polypeptide may be used for binding assays, for elucidation of signaling pathways involved in the determination of cytokine synthesis by T cells and macrophages, and for identifying ligands or substrates that bind to, modulate or mimic the action of CYTRF, that block CYTRF binding to its receptor, or that mimic CYTRF triggering of its receptor. Drug screening identifies agents that provide a replacement or enhancement for CYTRF function. Of particular interest are screening assays for agents that have a low toxicity for human cells. A wide variety of assays may be used for this purpose, including labeled *in vitro* protein-protein binding assays, protein-DNA binding assays, electrophoretic mobility shift assays, immunoassays for protein binding, and the like. The purified protein may also be used for determination of three-dimensional crystal structure, which can be used for modeling intermolecular interactions, transcriptional regulation, *etc.*

The term "agent" as used herein describes any molecule, *e.g.* protein or pharmaceutical, with the capability of mimicking or modulating the activity of CYTRF . Generally a plurality of assay mixtures are run in parallel with different agent concentrations to obtain a differential response to the various concentrations. Typically, one of these concentrations serves as a negative control, *i.e.* at zero concentration or below the level of detection.

Candidate agents encompass numerous chemical classes, though typically they are organic molecules, preferably small organic compounds having a molecular weight of more than 50 and less than about 2,500 daltons. Candidate agents comprise functional groups necessary for structural interaction with proteins, particularly hydrogen bonding, and typically include at least an amine, carbonyl, hydroxyl or carboxyl group, preferably at least two of the functional chemical groups. The candidate agents often comprise cyclical carbon or heterocyclic structures and/or aromatic or polyaromatic structures substituted with one or more of the above functional groups. Candidate agents are also found among biomolecules including peptides, saccharides, fatty acids, steroids, purines, pyrimidines, derivatives, structural analogs or combinations thereof.

Candidate agents are obtained from a wide variety of sources including libraries of synthetic or natural compounds. For example, numerous means are available for random and directed synthesis of a wide variety of organic compounds and biomolecules, including expression of randomized oligonucleotides and oligopeptides. Alternatively, libraries of natural compounds in the form of bacterial, fungal, plant and animal extracts are available or readily produced. Additionally, natural or synthetically produced libraries and compounds are readily modified through conventional chemical, physical and biochemical means, and may be used to produce combinatorial libraries. Known pharmacological agents may be subjected to directed or random chemical modifications, such as acylation, alkylation, esterification, amidification, *etc.* to produce structural analogs.

Where the screening assay is a binding assay, one or more of the molecules may be joined to a label, where the label can directly or indirectly provide a detectable signal. Various labels include radioisotopes, fluorescers, chemiluminescers, enzymes, specific binding molecules, particles, *e.g.* magnetic particles, and the like. Specific binding molecules include pairs, such as biotin and streptavidin, digoxin and antidigoxin *etc.* For the specific binding members, the complementary member would normally be labeled with a molecule that provides for detection, in accordance with known procedures.

A variety of other reagents may be included in the screening assay. These include reagents like salts, neutral proteins, *e.g.* albumin, detergents, etc that are used to facilitate optimal protein-protein binding and/or reduce non-specific or background interactions. Reagents that improve the efficiency of the assay, such as protease inhibitors, nuclease inhibitors, anti-microbial agents, *etc.* may be used. The mixture of components are added in any order that provides for the requisite binding. Incubations are performed at any suitable temperature, typically between 4 and 40°C. Incubation periods are selected for optimum activity, but may also be optimized to facilitate rapid high-throughput screening. Typically between 0.1 and 1 hours will be sufficient.

The compounds having the desired pharmacological activity may be administered in a physiologically acceptable carrier to a host for treatment of infection. Depending upon the manner of introduction, the compounds may be formulated in a variety of ways. The concentration of therapeutically active compound in the formulation may vary from about 0.1-100 wt.%.

The following examples are offered by way of illustration and not by way of limitation.

EXPERIMENTAL

The following examples are put forth so as to provide those of ordinary skill in the art with a complete disclosure and description of how to make and use the subject invention, and are not intended to limit the scope of what is regarded as the invention. Efforts have been made to ensure accuracy with respect to the numbers used (*e.g.* amounts, temperature, concentrations, *etc.*) but some experimental errors and deviations should be allowed for. Unless otherwise indicated, parts are parts by weight, molecular weight is average molecular weight, temperature is in degrees centigrade; and pressure is at or near atmospheric.

Materials and Methods

Animals. B10.A, B10.BR, and B10.PL mice, six to nine weeks old, were obtained from Jackson Laboratory (Bar Harbor, ME); (B10.AxB10.BR) F1; F2 and (B10.BRxF1) backcross mice were bred in our facility.

Antibodies. Anti-IL-4 mAb 11B11 was provided by J. O'Hara and W.E. Paul (NIH). Anti-IFN-γ mAb XMG1.2 was provided by T. Mosmann (University of Alberta, Edmonton, Canada). Anti-IFN-γ mAb HB170 was obtained from ATCC. Anti-IL-4 mAb BVD4 and BVD6, and anti-IL-10 mAb 2A5 and Sxc.1 were provided by M. Howard (DNAX, Palo Alto, CA). Anti-IL-12 p40 mAb C17.8 and C15.6 were provided by G. Trinchieri (Wistar Institute of Anatomy and Biology, Philadelphia, PA). Anti-TNF-α mAb was provided by Robert Schreiber (Washington School of Medicine, St. Louis, MO). Anti-IL-13 and anti-TGFβ pan-neutralizing antibodies were purchased from R&D Systems (Minneapolis, MN).

Peptides. Mouse MBP Ac1-16: Ac-ASQKRPSQRSKYLATA was purchased from the Protein and Nucleic Acids Facility (Stanford U., Stanford, CA).

T Cell Lines. Mice were immunized in the base of the tail with 100 μM mouse MBP Ac1-16 in CFA H37 Ra (Difco, Detroit, MI). Eight days later superficial inguinal and sacral lymph nodes were removed, and lymph node cells were stimulated with 30 μM mMBP Ac1-16 in RPMI 1640 (GIBCO BRL, Gaithersburg, MD) with 2% syngeneic mouse serum. Every 10-14 days thereafter 0.5-1x10^6 T cells were re-stimulated with 30 μM mMBP Ac1-16 and 5x10^6 irradiated (3000 rad) splenocytes per ml. Starting from the third re-stimulation T cell lines were cultured in RPMI 1640 with 10% FCS (GIBCO BRL, Gaithersburg, MD), and 10% supernatant from rat splenocytes activated overnight by 2 μg/ml of Concavalin A was added every other re-stimulation.

BR E7 T cell Clone. Every 7-10 days 2-5 x 10^5 T cells per ml were re-stimulated with 2-5 x 10^6 irradiated (3000 rad) B10.BR splenic APC per ml, 3 μg/ml KLH (Calbiochem, San Diego, CA) and 10% rat ConA supernatant. Culture supernatants were harvested 24 hours later and assayed for cytokines.

Induction And Scoring Of EAE. Three days after the last re-stimulation with 30 μM mMBP Ac1-16, 5x10^6 irradiated splenic APC, and IL-2, 2-5x10^7 T cells were washed three times in serum free RPMI and injected i.v. in the tail veins of non-irradiated recipient mice, 6-9 weeks old. Mice were monitored daily, and EAE severity was measured on the scale 0-5: 0, no symptoms; 0.5 straight tail; 1, limp tail; 2, paraparesis; 3, paraplegia; 4, quadriplegia; 5, moribund. Mice with score 4-5 were euthanized.

Induction of Cytokine Responses. At the time of re-stimulation aliquots of 0.25-1x10^6 T cells were washed and plated in 1 ml of RPMI 1640, 10% FCS. 30 μm mMBP Ac1-16, 5x10^6 irradiated (3000 rad) splenocytes and 10% rat ConA supernatant (if less than 1x10^6 T cells were used) were added for 24 hours, after which supernatant fluids were removed and assayed for levels of cytokines. For measuring effects of 2 hr supernatants on the levels of IFN-γ and TNF-α, supernatant fluids were obtained from culture of 1x10^6 B10.A and B10.BR T cells per ml re-stimulated with 30 μm mMBP Ac1-16 and 5x10^6 splenocytes per ml for 2 hours. Alternatively, 1 x

10^7 freshly isolated spleen cells per ml were incubated for 2 hours with 10 μg/ml LPS (E.Coli 0127:B8; Difco (Detroit, MI). The 2 hour supernatants from each strain (fresh or stored frozen at -80°C) were then used as culture media in which 0.5-1x10^6 B10.BR T cells per ml were re-stimulated with 5x10^6 irradiated B10.BR splenocytes per ml and 30 μm mMBP for 24 hours. In some experiments 5x10^5 T cells per ml were re-stimulated with 30 μm mMBP Ac1-16 and 5x10^5 per ml irradiated (10,000 rad) transfected L cell fibroblasts expressing A^k class II proteins (LA^k cells).

Depletion of Splenic T-cells. Spleen cells were dissociated in serum free RPMI 1640, resuspended at 4 x 10^7 per ml and incubated for 10 minutes at 4° with 100 μl/ml $(NH4)_2SO_4$-concentrated anti-Thy-1 mAb (Tib 99 from ATCC, Rockville, MD). Following incubation for 30 minutes at 37° with 1:8 diluted Low-Tox Baby Rabbit Complement (Cedarline, Westbury, NY), surviving cells were washed three times in RPMI 1640 with 10% FCS and analyzed by FACS with anti-CD3 mAb or with isotype-control hamster IgG (Caltag, Burlingame, CA).

Addition or Depletion of Cytokines. Cytokines were added as follows: mouse recombinant IL-4 (Boehringer, Indianapolis, IN) at 100 units/ml; mouse recombinant IL-12 (PharMingen, San Diego, CA) at 10 units/ml; mouse recombinant IFN-γ (Boehringer, Indianapolis, IN) at 100 ng/ml; mouse recombinant TNF-α (Genentech, South San Francisco, CA) at 2 ng/ml. To deplete cytokines from the 2 hour culture supernatants, anti-cytokine antibodies were added to 1 ml of 2-hour supernatants from B10.BR and B10.A cultures at the following concentrations: anti-IL-4 (11B11) at 10 μg/ml and 20 μg/ml; anti-IL-10 (Sxc.1) at 20 μg/ml and 40 μg/ml; anti-IL-12p40 (C17.8) at 20 μg/ml; anti-IL-13 (goat polyclonal) at 50 μg/ml and 75 μg/ml; anti-IFN-g (XMG1.2) at 10 μg/ml and 20 μg/ml; anti-TNF-α (TN3. 19.12) at 50 μg/ml and 100 μg/ml; anti-TGFb (rabbit polyclonal) at 40 μg/ml and 80 μg/ml. Following 30 min incubation at 4°C, 100 μl of protein G-coated agarose beads (Pierce, Rockford, IL) were added to the supernatants. After incubation for 30 min at room temperature, beads were removed from supernatants by centrifugation at 3000 rpm for 3 minutes, and supernatants were then used as a media for the re-stimulation of T cells.

ELISA. Double-antibody sandwich ELISA assays were used. Plates were coated overnight with a monoclonal antibody specific for a particular cytokine, washed and then incubated with culture supernatant or a cytokine standard overnight. A second monoclonal antibody, which was biotin-conjugated and which recognizes an independent epitope of the same cytokine, was used for detection. Antibodies were biotinylated with D-biotin-N- Hydroxysuccinimide ester (Sigma, St. Louis, MO). Biotinylated antibodies on the plate were detected with Streptavidin-HRP (Pierce, Rockford, IL) plus o-Phenylenediamine (Sigma, St. Louis, MO). Optical densities were determined at wavelength = 490 nm, using Molecular Devices (Sunnyvale, CA) plate reader. Anti-IL-4 mAb BVD4 or 11B11 was used for coating and BVD6 for detection of IL-4. Mouse recombinant IL-4 from DNAX (Palo Alto, CA) was used as a standard. Anti-IL-10 mAb 2A5

(coating) and Sxc.1 (detection) were used for IL-10. Mouse recombinant IL-10 from DNAX (Palo Alto, CA) was used as a standard. Anti-IL-12p40 mAb C17.8 (coating) and C15.6 (detection) were used to determine the IL-12 levels. Recombinant murine IL-12, provided by S. Wolf, Genetics Institute, (Cambridge, MA), was used as a standard. Anti-IFN-γ mAb HB170 (coating) and XMG1.2 (detection) were used for the IFN-γ ELISA. Recombinant murine IFN-γ (Genentech, South San Francisco, CA) was used as a standard.

IL-2 Bioassay. 50 μl of supernatants from T cell lines re-stimulated with serial dilutions of mMBP Ac1-16 and irradiated splenic APC for 24 hours were added to $1x10^4$ HT-2 cells per well in 96 well plates in a final volume of 200 μl. IL-4 was neutralized with 11B11 anti-IL-4 mAb. Murine recombinant IL-2 (Sigma, St. Louis, MO) was used as a standard. 16-18 hours later ^{3}H-thymidine (1 μCi per well in 20 μl) was added to plates. After 6 - 7 hours incubation plates were harvested (Ph.D. Cell Harvester), and incorporation of ^{3}H-thymidine was measured by scintillation counting.

TNF Bioassay. Briefly, $2x10^4$ L929 cells per well were plated in 96 well plates and incubated overnight. Serial dilutions of supernatants from T cell lines and 1 μg/ml actinomycin C1 (Boehringer, Indianapolis, IN) were added to wells in a final volume of 200 μl. A standard curve was prepared using recombinant murine TNF-α (Genentech, South San Francisco, CA). After 24 hours incubation 10 μl of 5 mg/ml MTT (Sigma, St. Louis, MO) was added to each well. 4 hours later 100 μl of solubilization solution containing 50% dimethylformamide and 20% SDS (Sigma, St. Louis, MO) were added to each well for 1 hour, and the optical densities were determined using a Molecular Devices (Sunnyvale, CA) plate reader (wavelength = 570 nm).

Results

T Cells from B10.BR Mice are Significantly More Encephalitogenic in Adoptive Transfer of EAE than B10.A-derived T Cells. Initial comparison of susceptibility of strains B10.A and B10.BR to EAE induced *in vivo* by immunization with MBP N-terminal peptides were uninformative, as both strains demonstrated comparably low susceptibility to EAE (average score 0.5). Freshly isolated B10.A and B10.BR lymph node cells gave very low proliferative responses to MBP compared to those from A^u strain B10.PL, which is susceptible to EAE. The differences in susceptibility to EAE and in proliferation of A^u - vs. A^k -restricted MBP-primed lymph node cells most likely reflects higher affinity binding of MBP N-terminal peptides by A^u compared to A^k class II proteins.

For adoptive transfer of EAE T cell lines (TCL) specific for mMBP Ac1-16 were generated from B10.PL, B10.A, B10.BR and (B10.AxB10.BR) F1 animals, using splenic APC in the serial re-stimulations. After several *in vitro* re-stimulations T cells were transferred into non-irradiated recipients using the EAE adoptive transfer model. In two adoptive transfer experiments two independently-derived sets of TCL specific for mMBP Ac1-16 (TCL 1 and TCL 2) were generated from B10.PL, B10.A, B10.BR and (B10.AxB10.BR) F1 animals. Strain B10.PL was used as a

positive control for the encephalitogenicity of MBP-specific TCL. The first set of TCL (TCL 1) was re-stimulated nine times *in vitro* and the second set of TCL (TCL 2) was re-stimulated five times *in vitro* prior to transfer into syngeneic recipients. TCL from B10.A and B10.BR strains were also transferred into (B10.AxB10.BR) F1 animals. The severity of EAE was scored on a standard scale from 0 to 5.

The results of these adoptive transfer experiments are presented in Table 1. The positive control B10.PL TCL induced severe EAE. Both TCL from B10.BR mice were also highly encephalitogenic in syngeneic recipients and caused disease in F1 recipients. In contrast, both B10.A-derived TCL failed to induce significant disease in either B10.A or F1 recipients. F1 T cells transferred into F1 mice induced weak EAE, though marginally more severe disease than that induced by B10.A T cells. These results show that B10.A and B10.BR strains display a significant phenotypic difference in that TCL from B10.BR induce severe EAE, whereas TCL from B10.A fail to do so. Low encephalitogenicity appears to be dominant in the (B10.A x B10.BR) F1 TCL specific for MBP.

Table 1. *MBP-specific TCL from B10.a or (B10.A x B10.BR) F1 Mice Are Less Encephalitogenic than MBP-specific TCL from B10.BR Strain*

Strain			Clinical signs of EAE		
				Maximum score	Day of onset
Donor TCL*	No.	Recipient mice	Incidence	Average (range)	Average (range)
B10.PL	TCL 1	B10.PL	5/5	1.9 (1-3)	7 (5-10)
B10.BR	TCL 1	B10.BR	5/5	2.7 (2-4)	10.6 (7-14)
	TCL 2	B10.BR	5/5	3.4 (3-4)	7.2 (6-10)
B10.BR	TCL 1	(A x BR)F1§	3/5	1.3 (0.5-2)	13.3 (8-17)
	TCL 2	(A x BR)F1	5/5	3.8 (3-4)	8.6 (7-10)
B10.A	TCL 1	B10.A	4/5‡	0.75 (0.5-1)	7 (4-12)
	TCL 2	B10.A	4/5	0.5 (0.5-1)	8 (7-15)
B10.A	TCL 1	(A x BR)F1	3/4	0.66 (0.5-1)	9 (6-11)
	TCL 2	(A x BR)F1	3/5	0.5	16 (12-20)
(A x BR)F1	TCL 2	(A x BR)F1	4/5	1 (0.5-1.5)	14.2 (9-20)

*TCL 1 cells were re-stimulated nine times in vitro before transfer, using syngeneic APC for B10.PL TCL and a random mixture of B10.A and B10.BR APC for B10.A and B10.BR TCL. TCL 2 cells were restimulated five times before transfer, using syngeneic splenic APC.

‡One out of five B10.A mice injected with B10.A T cells died shortly after the experiment, likely due to injury or stress.

§(A x BR)F1 is an abbreviation of (B10.A x B10.BR)F1.

The two B10.BR TCL differed in the severity of EAE induced in F1 recipients. B10.BR TCL 1, which had been re-stimulated with a random mixture of B10.BR and B10.A splenocytes, induced less severe EAE in (B10.A x B10.BR) F1 mice than did B10.BR TCL 2, which had been re-stimulated with B10.BR APC only. This suggested that the genetic origin of the APC influenced the encephalitogenicity of TCL. To test this hypothesis, additional TCL (TCL 3) were generated, in

which lymph node T cells from B10.A and B10.BR mice were re-stimulated as separate lines with B10.A, B10.BR, or (B10.AxB10.BR) F1 splenic APC. After nine re-stimulations with mMBP Ac1-16 presented by splenic APC of syngeneic or semi-syngeneic F1 genetic origin, the TCL were adoptively transferred into recipients. (TCL re-stimulated with the reciprocal parental APC did not expand sufficiently in number to transfer.) As shown in Table 2, neither B10.A TCL caused significant EAE, consistent with the results with TCL1 and TCL2. Strikingly, while B10.BR TCL re-stimulated with B10.BR splenic APC induced severe disease, the B10.BR TCL re-stimulated with F1 APC did not cause EAE. These results indicate that B10.BR splenic APC are required for the development of encephalitogenicity of B10.BR TCL and that the non-EAE-promoting phenotype of B10.A is dominant in F1 APC.

Table 2. *Encephalitogenicity of B10.BR MBP-specific T Cells Is Reduced by Re-stimulation with (B10.A x B10.BR)F1 Splenic APC*

Strain			Clinical signs of EAE		
				Maximum score	Day of onset
Donor TCL (TCL 3)	APC	Recipient mice	Incidence	Average (range)	Average (range)
B10.BR	B10.B	B10.BR	5/5	3.8 (3-4)	7.5 (5-10)
B10.BR	(A x BR)F1	B10.BR	1/4	0.5	9
B10.A	B10.A	B10.A	4/5	0.5	9 (6-12)
B10.A	(A x BR)F1	B10.A	3/5	0.5	12.5 (9-16)

Cytokine Profiles, but not Other Parameters Required for Encephalitogenicity, Differ among B10.A, B10.BR and (B10.A x B10.BR) F1 T Cell Lines. Encephalitogenic B10.BR TCL and non-encephalitogenic B10.A and F1 TCL were characterized for parameters previously shown to be required for the induction of EAE to determine which parameter is affected by the genetic differences between these strains. The activation state of TCL following stimulation with mMBP Ac1-16 was assayed by examining proliferation, cell surface levels of Mel-14, LFA-1, VEA (CD69), CD3, and CD4 antigens and by cell-cycle analysis. None of these indices of activation differed between the B10.A and B10.BR TCL; all TCL were virtually 100% $CD4^+$ and proliferated vigorously in response to MBP Ac1-16. The proliferative responses of all TCL were strictly mMBP Ac1-16-specific and dose-dependent whether re-stimulated with APC of syngeneic, F1, or reciprocal parent strain origin displayed no allogeneic reactivity. The potential for homing to the CNS was examined by measuring cell surface levels of $\alpha 4\beta 1$ integrin, VLA-4; no consistent differences were found between B10.A and B10.BR TCL. FACS and RT PCR analysis demonstrated that the predominant T cell receptor Vβ regions expressed by B10.A and B10.BR TCL were extremely similar (predominantly Vβ 4, 8, and 13), and somewhat more heterogeneous than that of the B10.PL MBP TCL (50% Vβ 8). The levels and sequences of the A^k class II presenting element for MBP Acl-16 were also examined for strains B10.A and B10.BR. By flow cytometry the levels of A^k expressed by splenic B cells, and splenic macrophages, and peritoneal

macrophages from B10.A and B10.BR mice were found to be identical. Splenic cDNA for $A\alpha^k$ and $A\beta^k$ chains were amplified from both strains and sequenced using direct PCR sequencing and were found to be identical over the entire protein coding sequence.

These results indicate that the class II presenting elements, mMBP Ac1-16-specific TCR repertoire, and indices of activation are identical between B10.A and B10.BR.

In contrast, comparison of the cytokine profiles of B10.A and B10.BR TCL revealed differences which correlated with their encephalitogenicity. Data for the TCL 2 lines are shown in Figures 1A-1F. Established lymph node T cell lines (TCL 2) from mMBP Ac1-16- immunized B10.A, B10.BR and (B10.AxB10.BR) F1 mice were re-stimulated for the fifth time with mMBP Ac1-16 and irradiated splenic APC. After 24 hours, culture supernatants were harvested. Additional cells harvested three days after the same fifth re-stimulation were used for adoptive transfer EAE. Data presented for TNF are means and standard deviations of two bioassays performed on triplicate wells for each sample (n=6). Data presented for IFN-g, IL-12, IL-4 and IL-10 are means and standard deviations of triplicate wells for each sample (n=3). Data presented for IL-2 are means and standard deviations of two IL-2 bioassays performed in duplicate wells for each sample (n=4).

The B10.A TCL stimulated with peptide and B10.A splenic APC displayed the Th2 phenotype, expressing very low levels of IFN–γ, IL-2 and TNF-α and high levels of IL-4 and IL-10. Levels of Th1-promoting IL-12 (made by APC) were undetectable. In contrast, the B10.BR TCL displayed the Th1 phenotype, secreting very little IL-4 and IL-10 and high levels of IFN-γ, IL-2 and TNF-α. IL-12 levels were also significantly higher. TNF-α ELISA results were nearly identical (within 10%) to the TNF bioassay results, and as TNF biological activity was totally blocked with the anti-TNF-α mAb TN3-19.12, all of the TNF activity detected in these TCL supernatants appears to be TNF-α. Supernatants from the (B10.AxB10.BR) F1 TCL stimulated with peptide and F1 APC contained intermediate amounts of the tested cytokines, but the levels were closer to those from B10.A than B10.BR TCL. Thus, TCL from B10.A mice produced a Th2, low TNF-α response, T cells from B10.BR produced a Th1, high TNF-α response, and the Th2, low TNF-α phenotype was dominant in the (B10.AxB10.BR) F1 T cell cultures. These results indicated that genetic difference between B10.A and B10.BR controls a regulator of Th cytokines.

The Cytokine Profile of the B10.BR TCL is Influenced by the Genetic Origin of the APC Used for in vitro Re-stimulation. The cytokine profiles of the third set (TCL 3) of MBP-specific T cell lines serially re-stimulated with splenic APC of varying genetic origin were also examined. The cytokine responses of the six TCL were determined after the third, fourth, fifth, ninth, and tenth re-stimulations. We found that the genetic origin of the splenic APC used for re-stimulations greatly influenced the cytokine profile of the B10.BR TCL. Thus, the Th1, high TNF-α phenotype

of the B10.BR TCL was maintained only by syngeneic B10.BR splenic APC; very little IFN-γ, TNF-α or IL-2 was produced by the B10.BR TCL serially re-stimulated with F1 or B10.A APC (Figure 2A-2E). Figure 2A-D: After the third, fourth, fifth, ninth and tenth re-stimulations of T cell lines with mMBP Ac1-16 and irradiated splenic APC of the indicated origin, T cells were re-stimulated for 24 hours with peptide and APC of the same origin. Adoptive transfer of EAE was performed after the ninth re-stimulation (See Table 2). Supernatant fluids were analyzed for cytokines. In Figure 2 D the points for the B10.BR TCL 3 - B10.A APC are 37 ng/ml of IL-10 after the ninth re-stimulation and 45 ng/ml of IL-10 after the tenth re-stimulation. Data presented are average of duplicate wells for each sample. Figure 2E At the ninth re-stimulation TCL 3 described for panels A-D were re-stimulated for 24 hours with peptide and APC of the same (indicated) origin. Supernatant fluids depleted of IL-4 were analyzed for IL-2 levels, using the growth factor dependent cell line HT-2. Data presented are means and standard deviations of four wells for each sample (n=4).

For IL-4, all three of the B10.A TCL had higher levels than the B10.BR TCL (Figure 2 C), and B10.BR T cells maintained on B10.BR APC produced the lowest IL-4 levels. For IL-10, B10.A and F1 APC, but not B10.BR APC supported levels similar to those produced by B10.A TCL, (Figure 2 D). A Th2, low TNF-α profile was seen for all B10.A TCL irrespective of the source of APC.

Expression of the Th1, high TNF-α phenotype only by B10.BR TCL serially re-stimulated with B10.BR APC correlates well with the encephalitogenicity of this TCL in adoptive transfer of EAE (Table 2). These results indicate that B10.A and F1 APC prevent the development of the Th1, high TNF-α encephalitogenic cytokine phenotype in response to MBP.

IFN-γ and TNF-α Production by Established Th1 B10.BR TCL can be Down-regulated by a Single Re-stimulation with B10.A APC or in the Presence of 2-hour Supernatants from Activated B10.A Cultures. To determine whether a single re-stimulation with B10.A APC can alter the production of IFN-γ and TNF-α by established B10.BR-derived Th1 cells, two independently-derived Th1 MBP-specific B10.BR TCL (TCL 3 and TCL 4, both of which had been maintained with syngeneic B10.BR APC) were re-stimulated overnight with B10.BR or B10.A APC prior to harvesting supernatants for cytokine assays. As shown in Figure 3A, B and C (top bars), IFN-γ and TNF-α levels were down-regulated by 40-60% when B10.A instead of B10.BR APC were used for the single re-stimulation. Figure 3 A, B and C represent independent experiments with MBP-specific B10.BR TCL 3 (A and B) and TCL 4. (C) TCL were re-stimulated for 24 hours in the presence of B10.BR or B10.A splenic APC, or splenic APC depleted of T cells (panel A, lower bars). In some cultures B10.BR TCL were re-stimulated with B10.BR APC and MBP peptide in the presence of 2-hour supernatant fluids from cultures of B10.BR or B10.A TCL stimulated with syngeneic APC and MBP peptide (Figure 3 B, bottom bars; 3 C, middle bars); or in the presence

of 2-hour supernatants from B10.BR or B10.A spleen cells stimulated with LPS (Figure 3 C, bottom bars). Data presented are means and standard deviations of triplicate wells for each sample (n=3). All experiments were repeated at least three times with similar results.

Similar down-regulation of IFN-γ and TNF α was observed when B10.A (but not B10.BR) T cell-depleted splenic APC were used to re-stimulate B10.BR TCL (Figure 3 A, bottom bars). Irrespective of the genetic source of APC IL-2 levels and proliferative responses remained high and IL-4 was not induced.

To determine whether the inhibitory activity of B10.A APC on IFN-γ and TNF-α levels was mediated by a soluble factor, the two B10.BR TCL were re-stimulated with mMBP Ac1-16 and B10.BR splenic APC in the presence of supernatant fluids from a culture of B10.A TCL stimulated with B10.A splenic APC and mMBP Ac1-16 for 2 hours. The 2-hour time point was chosen because most cytokines induced by activation of T cells and APC are not detectable at this time, except TNF-α and -β. The B10.A culture-derived 2-hour supernatant down-regulated IFN-γ and TNF-α production by at least 50% relative to control cultures with 2-hour supernatants from B10.BR T cells and APC plus peptide (Figure 3B, bottom bars and 3C, middle bars), comparable to the effects of B10.A APC themselves. Similar results were obtained with B10.BR TCL 4 stimulated with B10.BR splenic APC and MBP peptide in the presence of supernatants from freshly-isolated B10.A and B10.BR spleen cells activated for 2 hours with LPS, (Figure 3C, bottom bars). Thus, down-regulation of IFN-γ and TNF-α production seems to be mediated by a soluble factor secreted by B10.A splenic APC within the first 2 hours after activation by antigen-stimulated T cells or by LPS. However, a single re-stimulation of B10.BR TCL with B10.A splenic APC or in the presence of 2-hour supernatant from B10.A cultures did not induce any IL-4 expression in B10.BR T cells. B10.A TCL remained Th2, low TNF-α producing cells irrespective of the genetic source of APC or 2 hour supernatant.

The B10.A-derived Soluble Cytokine Regulatory Factor Functions when A^k-expressing L-cell Fibroblasts are Used as APC. To begin to clarify the target of action of the B10.A-derived regulatory factor, B10.BR or B10.A T cells (TCL 4) were re-stimulated twice with mMBP Ac1-16 presented by non-professional APC (A^k-expressing L cells which do not produce splenic APC-derived cytokines), in the presence of 2-hour supernatant from B10.A cultures (Figures 6A-6B). In either normal medium or in the 2-hour B10.BR supernatant, B10.BR T cells produced high levels of IFN-γ and TNF-α, and B10.A T cells produced high levels of IL-4 with little or no TNF-α and IFN-γ. However, the levels of IFN–γ and TNF-α produced by B10.BR TCL were reduced in the presence of 2-hour supernatant from B10.A cultures, similar to the effects seen when professional splenic APC were used (Figures 4A-4C). This result indicates that B10.A-derived factor does not require the presence of splenic APC for its action and probably acts directly on the responding T

cells. B10.BR (top bars) or B10.A (bottom bars) TCL 4 were activated with mMBP Ac1-16 and A^k-expressing L cell fibroblasts as APC for two consecutive re-stimulations in either normal medium or in 2-hour supernatants from B10.BR or B10.A T cells activated with syngeneic splenic APC. Culture supernatants were harvested 24 hours post re-stimulation and IFN-γ, TNF-α and IL-4 were measured as above. Data presented are means and standard deviations of triplicate wells for each sample (n=3). Similar results were obtained in two additional experiments.

The B10.A-derived Factor Acts Independently of Known Th1/Th2 Regulatory Cytokines in Down-regulating B10.BR Production of IFN-γ and TNF-α. To assess whether down-regulation of IFN-γ and TNF-α by B10.A APC is mediated by or depends on any of the known Th1/Th2 regulators, 2-hour culture supernatants derived from B10.BR or B10.A TCL/APC cultures were depleted of IL-4, IL-10, IL-12 p40, IL-13, TNF-α, IFN-γ and TGF-β and tested for their effects on IFN-γ and TNF-α production by B10.BR TCL4 re-stimulated with B10.BR APC and mMBP peptide. Cytokine depletion did not diminish the down-regulatory activity of the B10.A culture supernatant was observed (Figure 5 A and B). Serial dilutions of B10.A and B10.BR 2-hour culture supernatants (Figure 5 C) demonstrated that the down-regulatory activity of B10.A-derived factor is rapidly lost by dilution, indicating that any depletion of the down-regulatory cytokine would have been detected. Essentially identical results were obtained when the anti-cytokine antibodies were also used at half the concentrations for which data are shown in Fig. 5.

Figures A and B: B10.BR or B10.A T cells (TCL 4) were re-stimulated with mMBP Ac1-16 and syngeneic splenic APC for 2 hours. Neutralizing antibodies specific for mouse IL-4, IL-10, TNF-α, IFN-γ, TGF-β, IL-13 and IL-12p40 were added to these culture supernatants for 30 minutes, and antibody-cytokine complexes were removed using protein G-coated agarose beads. Cytokine depleted 2-hour supernatants were then used as media in which B10.BR TCL 4 were re-stimulated with mMBP Ac1-16 and B10.BR splenic APC for 24 hours. IFN-γ (panel A) and TNF-α (panel B) levels were measured as above. Lower levels of TNF-α following treatment with anti-TNF-α antibody and protein G beads were due to the presence of residual anti-TNF-α antibody, which has low affinity for protein G in medium with serum. Neutralizing antibodies were used at concentrations equal to and twice recommended by the manufacturers for complete neutralization of cytokine levels produced by high expressing cells, giving similar results. Data presented, for the higher antibody concentration, are means and standard deviations of triplicate wells for each sample (n=3). Figure 5C: B10.BR TCL-4 cells were re-stimulated with B10.BR splenic APC and mMBP Ac1-16 in 2-hour culture supernatants from B10.A or B10.BR TCL-4, used either undiluted or at serial 1:2 dilutions in RPMI, 10% FCS. TNF-α levels are means and standard deviations of triplicate wells for each sample (n=3).

As summarized in Figures 6A-6B, blocking or adding IL-4 and IL-12 (alone or in combinations) did not reduce the down-regulatory effects of B10.A APC on TNF-α levels, although there were effects on the absolute levels of TNF-α production. B10.BR T cells (TCL 4) were re-stimulated with mMBP Ac1-16 and either B10.BR or B10.A splenic APC for 24 hours in the absence or presence of mouse recombinant IL-4, IFN-γ, TNF-α and IL-12 alone or combination with neutralizing antibody specific for mouse IL-12, or mouse IL-4. Cytokine levels resulting from each treatment are expressed as % of control (B10.BR TCL, B10.BR APC and peptide with no cytokine or antibody additions). Data presented are means and standard deviations of triplicate wells for each sample (n=3). At least two independent experiments were performed for each treatment, yielding similar results. In contrast, adding IFN-γ significantly reduced the down-regulation by B10.A APC. For IFN-γ production, adding TNF-α did not prevent the down-regulation of IFN-γ by B10.A APC, and adding IL-4 did not synergize with this down-regulation (Figure 6 B). As expected because of their pro-Th1 effects, addition of IL-12 alone, or in combination with anti-IL-4, did increase the overall IFN-γ levels and reduced the down-regulation of IFN-γ by B10.A APC.

In the experiments shown in Figures 5 and 6, none of the cytokines or anti-cytokine antibodies induced detectable levels of IL-4 production by the B10.BR TCL. Overall the results suggest that the B10.A-derived factor acts independently of known cytokine regulators, and that strong pro-Th1 conditions can overcome its down-regulatory effect on TNF-α and IFN-γ.

B10.A APC Down-regulate IFN-γ and TNF-α Production by a Th1 T Cell Clone with Different Antigen and MHC Restriction Specificities. The cytokines produced by the B10.BR-derived KLH-specific, E^k-restricted Th1 clone BR E7 were assayed following two consecutive re-stimulations with B10.BR or B10.A splenic APC. As shown in Figure 7, presentation of KLH by B10.A APC results in the progressive reduction of IFN-γ and TNF-α levels produced by this Th1 clone. Thus, the B10.A APC-derived factor acts independently of the specificity of the Th1 cell and is able to down-regulate IFN-γ and TNF-α production by a fully-committed clonal Th1 cell population. The BR E7 T cell clone was activated with KLH and either B10.BR or B10.A splenic APC for two consecutive re-stimulations. Culture supernatants were harvested and assayed for TNF-α (Figure 7A) and IFN-γ (Figure 7B) 24 hours post stimulation. Data presented are means and standard deviations of triplicate wells for each sample (n=3). Similar results were observed in at least two additional experiments.

A genetic difference(s) between inbred strains B10.A and B10.BR act(s) in APC to control Th1/Th2 cytokine profiles and the encephalogenicity of MBP-Ac1-16-specific T cells. The

regulatory effects are manifested both at the time of commitment to Th1/Th2 phenotypes, as B10.A APC block the development of IL-2, IFN-γ, and TNF-α production, and in the production of IFN-γ, and TNF-α by committed Th1 cells, as a single re-stimulation with B10.A-derived APC or in the presence of B10.A-derived supernatant down-regulates the production of these cytokines by established Th1 B10.BR cells. The down-regulation of IFN-γ, and TNF-α production by a B10.BR-derived KLH-specific, E^k-restricted T cell clone when re-stimulated by KLH presented by B10.A APC demonstrates that the regulatory effects of B10.A-derived factor 1) are not limited to responses of MBP-specific, A^k-restricted Th1 cells, and 2) can act by affecting individual clonal populations of mature Th1 cells rather than by expanding uncommitted precursors or Th0 cells. The induction of comparable proliferative and IL-2 responses in Th1 B10.BR TCL by MBP peptide presented by either B10.A or B10.BR APC indicates that B10.A APC do not inhibit the activation of these T cells but down-regulate production of IFN-γ and TNF-α. The target of the factor appears to be the responding T cells, as the down-regulatory effects of the B10.A supernatants are observed when MBP peptide is presented to B10.BR T cells by non-professional APC, A^k-expressing L cell fibroblasts. B10.A TCL maintain a Th2, low TNF-α cytokine profile irrespective of the genetic source of the APC or 2-hour supernatant, suggesting that they had become committed *in vivo*, perhaps due to the pro-Th2 effects of their own APC. The Th2-inducing phenotype of B10.A is dominant in (B10.A x B10.BR) F1 cells.

The identity of the APC cell type(s) within the irradiated B10.A splenocyte population that is responsible for the down-regulation of IFN-γ and TNF-α production has not yet been determined. Splenic T cells seem not to be required for the down-regulation. Preliminary results indicate that B10.A-derived splenic macrophages have down-regulatory activity, while splenic B cells do not. The results to date indicate that the down-regulation of IFN-γ and TNF-α production by B10.BR Th1 cells is mediated by B10.A APC, but it is possible that activated B10.A T cells also produce the down-regulatory factor.

The B10.A-derived soluble factor that modulates Th cytokine production is novel. The results of adding or blocking cytokines implicated in regulating Th1/Th2 cytokine production indicate that the down-regulatory effects of this factor probably do not act through or require IL-4, IL-10, IL-13, IL-12p40, TGF-b, IFN–γ and TNF–α. The B10.A-derived factor also acts independently of prostaglandin E2, an APC-derived molecule known to inhibit production of IFN–γ by activated CD4+ T cells, indicated by the failure of indomethacin to block down-regulation of IFN–γ and TNF–α production by B10.A APC. The activity of this factor in down-regulating IFN-γ production also differs from that of IL-4 in that IL-4, but not the factor, blocks IFN–γ induction by IL-12 through its inhibition of signalling through the IL-12 receptor.

The finding that the down-regulatory effects of the B10.A-derived factor on Th1 IFN–γ production is overcome by exogenous IFN–γ and by IL-12, especially in combination with anti-IL-4,

suggests that the *in vivo* effects of the B10.A-derived factor should be most evident in the absence of strong pro-Th1 conditions. This may occur during initial antigen priming, especially when T cell responses are weak - for example, with poor antigens such as the MBP Ac1-16 peptide, and perhaps more generally with self antigens. The apparent direct effect of B10.A APC and of the secreted cytokine regulatory factor on IFN–γ production by mature Th1 cells, even when IL-4 has been depleted, suggests that inhibition of IFN–γ production may be the primary mechanism by which the B10.A-derived factor skews initial Th1/Th2 subset determination. When T cells are strongly activated, or where significant levels of IL-12 and/or IFN–γ are already present, the B10.A-derived factor may not down-regulate IFN–γ production. Unlike IFN–γ, down-regulation of TNF–α is maintained in the presence of exogenous IL-12, implying IFN–γ and TNF–α are regulated independently.

The basis for the difference between B10.A and B10.BR APC in down-regulatory factor production remains to be determined. The gene for the factor itself could be polymorphic, resulting in differences in the levels or activity of the factor produced. Alternatively, the polymorphism could be in gene(s) that indirectly affect factor activity, by controlling its synthesis, modification, or secretion. Initial analysis of the genetics down-regulatory activity have yielded surprising results. It was anticipated that the gene would be *H-2*-linked, as B10.A and B10.BR are *H-2* congenic strains on the C57BL/10 background: the $H\text{-}2^k$ haplotype of B10.BR was derived from C57BR and the $H\text{-}2^a$ recombinant haplotype of B10.A (*k* haplotype in the *H-2K-Ea* region, *d* haplotype telomeric to *Ea*) was derived from strain A/WySn. However, preliminary tests of (B10.A x B10.BR) F2 progeny and (F1 x B10.BR) backcross progeny, in which their splenic APC were assayed for the ability to down-regulate TNF–α production by MBP-specific B10.BR TCL, suggest that the polymorphic gene(s) controlling factor production is/are not linked to *H-2*. If confirmed, this would indicate that strains B10.A and B10.BR also differ by a non-*H-2* locus or loci. Splenic APC from A strain subline A/WySn and from the A strain background *H-2*congenic A.TL down-regulate IFN–γ and TNF–α production by B10.BR TCL in response to MBP Ac1-16. Thus the production of the down-regulatory factor by B10.A appears to be due to a contaminating non-*H-2* gene(s) derived from the A/WySn donor of the $H\text{-}2^a$ haplotype, despite the ten backcrosses to the C57BL/10 inbred partner during the initial derivation of B10.A.

The genetic and phenotypic properties of the B10.A-derived factor clearly distinguish it from another major polymorphic gene previously shown to regulate Th1/Th2 cytokine production. The *Leishmania* resistance gene acts in T cells to regulate their Th1/Th2 cytokine phenotypes, whereas the results reported here show that the B10.A-derived factor acts in the APC used for T-cell activation (Howard *et al.* (1980) Parasite Immunol. 2:303-314). Also, for *Leishmania*, most inbred strains, including B10.A and A strains, have the resistant (Th1) phenotype. Finally, in the *Leishmania* system the resistant (Th1) phenotype is largely dominant in heterozygotes, whereas

for the B10.A-derived factor discussed here the Th2-promoting phenotype is dominant or co-dominant in (B10.A x B10.BR)F1 mice (Seder *et al.* (1992) J.E.M. **176**:1091-1098).

This gene represents a new member of a growing number of polymorphic genes that influence susceptibility to autoimmune diseases. Identification of the soluble cytokine regulatory factor controlled by the B10.A-derived gene, its cellular origin, and its mode of action, contribute to deciphering the mechanisms regulating Th1 and Th2 cells and their pathogenicity in autoimmune, inflammatory, and allergic disorders.

Example 2

Characterization of the Cytokine Regulatory Factor and its Activities

B10.A APC produce a soluble factor that down-regulates production of TNF-α and IFN-γ by Th1 cells. B10.BR APC either make no or lower levels of this factor, or at reduced activity. This soluble molecule, cytokine regulatory factor (CYTRF), is herein characterized in terms of both its biological activity and its biochemical properties. The screening assay used in the characterization of CYTRF was based on the fact that in a single stimulation of Th1 cell lines or clones B10.A APC or B10.A-derived culture supernatant reduces production of TNF-α and IFN-γ compared to APC or culture supernatant from strain B10.BR. The results suggest that CYTRF reduces the magnitude of the Th1 response to weak and/or self antigens. Studies with MBP and Hb peptide analogues provided herein demonstrate that the down-regulatory effects of CYTRF are inversely related to the strength of signaling via TCR, and that when T cell stimulation is very strong, B10.A and B10.BR APC support similar levels of TNF-α and IFN-γ.

To test this hypothesis directly, two experimental systems have been used, in which Th1 cells are stimulated by antigenic peptides that bind 1) with different affinity to MHC class II (MBP system), or 2) with different affinities to the TCR when complexed to MHC class II (hemoglobin (Hb) system). It has been previously established that an MBP analogue with lysine at position 4 substituted by glutamic acid, MBP Ac 1-16(4E), binds at least 100X stronger to A^k than the wild type peptide (Tate *et al.* (1995) *Intl. Immunol.* **7**,747-761; Lee *et al.* (1998) J Exp Med. **187**(9):1505-1516). Such differential affinity reflects the nature of the polymorphic amino acid residue at position 9 in the class II A_β chain. As expected, glutamic acid instead of lysine in peptide position 4 was also shown to result in a significantly lower dose of peptide required for activation of T cells specific for the N-terminal MBP peptides.

The second experimental system utilizes a panel of peptide analogues derived from the Hb-64-76 peptide (Hb-β^d allele), which is presented by I-E^k, and has been tested for their antigenic properties using a specific T cell clone, 3L2 (Sloan-Lancaster *et al.* (1994) *Cell* **79**, 913-922). It has been shown that position 72 (N in the wild type Hb) is a TCR contact site and is important for

T cell activation; substitutions at this site do not affect binding affinity to class II E^k. Amino acid substitutions at position 72 produced a set of altered peptide ligands with partially agonistic and antagonistic properties. For some, but not all Hb analogs, the differences in the T cell responses were linked to qualitatively-different signal transduction downstream of TCR, manifested by distinct patterns of tyrosine phosphorylation of ZAP-70 and CD3ζ chains (Kersh *et al.* (1996) *J. Exp. Med.* **184,** 1259-1268). Similar studies using altered peptide ligands were also performed by other research groups, with the general conclusion that a higher state of CD3ζ phosphorylation and of ZAP-70 association with CD3ζ chain stimulated by strong agonist peptides results in more pronounced Th1 response (Constant and Bottomly (1997) *Annu. Rev. Immunol.* **15,** 297-332).

CYTRF was also analyzed for its effects on cytokine production by macrophages. COnsistent with its activity in inhibiting production of Th1 cytokines IFN-γ and TNF-α, CYTRF inhibits production of the pro-Th1 cytokine IL-12 by macrophages.

Materials and Methods

Origin, maintenance, and stimulation of T cells. The B10.BR-derived MBP-specific T cell line TCL-4 was generated as described above. TCL-1s lines were established from B10.A and B10.BR mice by two consecutive immunizations with mMBP Ac1-16, following by isolation and serial re-stimulations of spleen cells from the primed animals. After 3 - 4 *in vitro* serial re-stimulations both B10.A and B10.BR strains generated mMBP Ac1-16-specific T cell lines producing Th1 cytokines. All mMBP-specific TCL were maintained by antigenic re-stimulations every 10 - 14 days. The 3L2 T cell clone was maintained by 14 day re-stimulations with 1 μM wild type Hb presented by B10.BR lethally-irradiated splenic APC, in the presence of 0.2 U/ml mouse recombinant IL-2 (Sigma).

Peptides and antibodies used for functional assays of T cell responses. Mouse MBP Ac1-16 and mouse MBP Ac1-16(4E) were synthesized by the Stanford Protein and Nucleic Acid facility (Stanford, CA); wild type Hb 64-76 and Hb with substitutions of threonine, Hb(T), and isoleucine, Hb(I) at position 72 were either obtained from P. Allen's laboratory or were synthesized by the Stanford Protein and Nucleic Acid facility. Anti-CD4 antibody (L3/T4 rat anti-mouse IgG2b) was purchased from PharMingen.

Purification of splenic macrophages and B cells. Spleens were freshly dissociated in cold complete RPMI (RPMI 1640; 10% FCS), and erythrocytes were removed by a Ficoll gradient (Histopaq, Sigma). Cells from the Ficoll interface were washed two times in PBS, 2% FCS and reacted with 1 μg/ml macrophage-specific F4/80-biotinylated antibody (monoclonal rat anti mouse

IgG2b, Caltag) in the same buffer for 20 minutes at 4°C. Then macrophages were purified with streptavidin-coated magnetic beads (Miltenyi, Auburn, CA) according to the Miltenyi protocol. The flow through F4/80 negative cells were then purified according to the Miltenyi protocol using magnetic beads coated with goat-anti-mouse IgG antibodies, selecting B cells. Macrophages were purified by the overnight adherence in complete RPMI. The purity of macrophage and B cell populations was assayed by FACS, using Mac-1 and B220 antibodies (both monoclonal rat anti-mouse IgG2b and IgG2a, respectively, from Caltag), and was found to be 98% for both cell types.

Induction of IL-12 and TNF-α production by macrophages. Resident peritoneal macrophages were harvested by peritoneal lavage, plated at 2.5 -5 x 10^5 cells/ml, and purified by adherence for 1 hour in complete RPMI. Then macrophages were treated with 20 μg/ml LPS (*E.coli*, 0127:B8, Sigma) and 100 U/ml mouse recombinant IFN-γ (Sigma). Culture supernatants were collected at 2 hours and at 24 hours, and the levels of IL-12 and TNF-α were measured as described above.

Biochemical characterization of CYTRF.

FPLC fractionation: B10.A 2 hour culture supernatant was prepared, as described above, but was derived from Th1 B10.A TCL-1s. After 10X concentration with Centriplus 100 column (molecular weight cut off of 97 kD, Amicon, Beverly, MA), retained proteins were separated on an FPLC Superose 6 column (Pharmacia FPLC, Pinscataway, NJ). Molecular weight calibration of FPLC column was determined with chymotrypsin, ferritin, thyroglobin, and blue dextran standards. FPLC fractions were then separately tested for their ability to down-regulate TNF-α production by BR E7 Th1 clone, as described above. Complete RPMI and FPLC buffer were used as negative controls.

Trypsin digest: B10.A TCL-1s and B10.BR TCL-1s 2 hour culture supernatants were treated with trypsin/EDTA (GIBCO) for 2 hours at 37°C. Then supernatants were concentrated with Centriplus 100 columns and complete RPMI was added to block trypsin, after which another Centriplus 100 concentration was performed. Trypsin-digested, concentrated 2 hour culture supernatants were treated with 0.1 mM PMSF (Sigma) and their ability to down-regulate TNF-α was tested on the B10.BR TCL-4 Th1 line, as described. Untreated 2 hour culture supernatants processed similarly were used as a positive control.

DE52 and Concanavalin A separation: B10.A TCL-1s and B10.BR TCL-1s 2 hour culture supernatants concentrated with Centriplus 100 were absorbed with DE52 beads (Sigma) in low pH, low salt buffer (20 mM TrisHCl, 50 mM NaCl), or with concanavalin A beads (type IV, Sigma) in PBS modified according to Sigma instructions. Proteins bound to the DE52 beads were eluted

with 0.5 M NaCl, 20 mM Tris-HCl; proteins bound to the concanavalin A beads were eluted with 100 mg/ml α-glucose. After concentration and dialysis into RPMI 1640, flow through and bound fractions were tested for their ability to down-regulate TNF-α production by B10.BR TCL-4 Th1 cells.

CyCAP: depletion, purification and addition, and metabolic labeling.

Depletion: B10.A TCL-1s and B10.BR TCL-1s 2 hour culture supernatants concentrated with Centriplus 100 were depleted of CyCAP by adding 1:250 final dilution of rat anti-CyCAP antiserum, with subsequent removal of the CyCAP-antibody complexes by a 1.5 hour incubation with protein A beads (Pierce) at 4°C. In a separate experiment Centriplus 100-concentrated 2 hour culture supernatants were incubated for 3 hours at 4°C with the glutathione beads (Sigma) coated with Cyp C-GST fusion protein.

Purification and Addition: 3×10^7 thioglycollate-elicited peritoneal lavage cells from 3 mice were plated at 1×10^6/ml in complete RPMI and purified by adherence. Macrophage culture supernatants harvested after one hour adherence and peritoneal fluids from which the macrophages were obtained were concentrated with Centriplus 100, and CyCAP was purified by binding to the Cyp C-GST-coated glutathione beads and subsequent elution with 30 μg/ml CsA, as previously described (Friedman *et al.* (1993) *Proc. Natl. Acad. Sci. USA* **90,** 6815-6819). Eluted CyCAP (and 30 μg/ml CsA used as a negative control) were then dialyzed in PBS overnight and further concentrated with Centricon 100 spin filters.

Metabolic labeling: Thioglycollate-elicited peritoneal lavage cells were cultured at 3×10^6 cell/ml in methionine-free, cysteine-free RPMI 1640 (Sigma), 2.5% FCS for 1 hour, after which non-adherent cells were removed and 0.5 mCi/ml of S^{35}-methionine (Amersham) was added to the macrophages. After four hours of the metabolic labeling, culture supernatants were harvested, concentrated with Centricon 100 columns, and CyCAP was purified with the Cyp C-GST-coated glutathione beads and eluted with CsA, as above. CyCAP was then resolved on a 10% SDS-PAGE protein gel.

CyCAP-specific RT PCR. Total RNA was extracted with RNeasy kit (Qiagen) from 1×10^6 thioglycollate-elicited peritoneal macrophages at 0, 1, 2 and 24 hours post isolation, according to the Qiagen protocol. 100 μl cDNA was synthesized from 1 μg of RNA, using oligo dT primers and MMLV reverse transcriptase (GIBCO), as recommended by GIBCO. Then RT PCR was performed with Taq polymerase (GIBCO) and CyCAP-specific primers: [SEQ ID NO:5] 5' AT G GCT CTC CTG TGG CTC CTC TC, 3' [SEQ ID NO:6] CAC CAT GTC AGT GGA GTT AGT GAG. The following conditions were used for the amplification: 2 min at 95°C, 35 amplification cycles of 1

min at 94°C, 1 min at 58°C, 3 min at 72°C, then 10 min at 72°C. Amplified cDNA was resolved on 1% agarose gel containing 0.5 μg/ml ethidium bromide, and visualized using digital camera.

Results

The B10.A-derived cytokine regulatory activity is produced by macrophages. B10.BR mMBP-specific Th1 TCL-4 was stimulated with mMBP Ac1-16 presented by pure populations of splenic mature macrophages or B cells or by unfractionated splenic APC. Unfractionated splenic APC and macrophages displayed the B10.A-derived down-regulatory activity, as levels of TNF-α produced by the TCL-4 were reduced by about 70% when purified APC were derived from the B10.A strain. In contrast, B10.A-derived B cells showed significantly diminished capacity to down-regulate TNF-α production, which was inhibited only by 20% compared to the B10.BR APC (Figure 8). 2×10^5 TCL-4 cells/ml were stimulated with 30 μM mMBP Ac1-16 presented by B10.A or B10.BR APC as follows: 1) 2×10^6 unsorted splenic APC, 2) 2×10^5 macrophages, 3) 8×10^6 B cells. Culture supernatants were harvested 24 hours later and assayed for TNF-α. Presented are the means and standard deviations of triplicate wells of TNF-α bioassay.

These results indicated that the cytokine regulatory factor mediating the genetic difference between the B10 congenics is produced by macrophages. Therefore, subsequent experiments based on the comparison of Th1 down-regulatory activity in B10.A and B10.BR strains were performed mostly with pure peritoneal macrophages. These experiments confirmed that B10.A macrophages are responsible for the Th1 inhibitory activity.

CYTRF is a glycosylated protein of about 100 kD. To characterize the nature of CYTRF, several biochemical characterizations were performed with two-hour culture supernatants from B10.A or B10.BR TCL-1s (both producing Th1 cytokines but differing in CYTRF activity) stimulated by syngeneic APC. These 2 hour culture supernatants were digested with trypsin, or incubated with anion-exchange resin DE52 or with concanavalin A-coated beads, followed by elution of bound proteins. Figure 9A shows levels of TNF-α_produced by B10.BR Th1 cells stimulated with mMBP Ac1-16 and B10.BR APC in the presence of control or treated B10.A or B10.BR two hour culture supernatants. 5×10^5 TCL-4 cells were stimulated with 30 μM mMBP Ac1-16 and 5×10^6 B10.BR splenic APC in either B10.A or B10.BR two-hour culture supernatants (unmanipulated, treated with trypsin, absorbed by or eluted from the DE52 or Concanavalin A beads, as indicated). Shown are means and standard deviations of triplicate wells of the TNF-α bioassay; similar data were obtained in an additional experiment.

The B10.A-derived down-regulatory activity was destroyed by trypsin and was absorbed either by DE52 in the low salt buffer, or by the concanavalin A-coated beads. The Th1 down-

regulatory activity of the B10.A two-hour culture supernatant was present in the DE52 0.5 M NaCl eluent or in the concanavalin A eluent. These results demonstrate that CYTRF is a glycosylated acidic soluble protein.

Comparison between B10.A and B10.BR two-hour culture supernatants demonstrated that B10.A-derived down-regulatory activity was present in the top, and not in the bottom fraction of the Centricon 100 protein concentrator, which has the molecular weight cut off of about 97 kD. Following this result, B10.A two-hour culture supernatant was fractionated on FPLC, and the CYTRF activity of fractions with different molecular weight was tested as above for their ability to down-regulate production of TNF-α by Th1 cells. As shown in Figure 9B, the down-regulatory activity was present in several fractions ranging from 100 kD to 470 kD, with the most prominent inhibition of the TNF-α production mediated by the 100 kD fraction. 5×10^5 BR E7 cells were stimulated with 10 μM KLH and 5×10^6 B10.BR splenic APC in the indicated FPLC fractions derived from two-hour B10.A culture supernatant. The RPMI line corresponds to the levels of TNF-α produced in complete RPMI medium. Addition of the FPLC buffer to the stimulated BR E7 cells did not reduce levels of TNF-α. Presented are the means of duplicate wells of TNF-α bioassay with variance within 15% of the mean. Thus, the Th1 inhibitory protein has a molecular weight of approximately 100 kD or higher.

The B10.A-derived CYTRF activity is associated with CyCAP. CyCAP was considered a candidate for the activity of CYTRF because of its similarity in size and secretion by macrophages. To assay whether CyCAP participates in the observed down-regulation of the Th1 cytokine production, two approaches were used. CyCAP-depleted two-hour B10.A or B10.BR culture supernatants were tested for CyCAP activity. Alternatively, purified B10.A-derived CyCAP was added during the activation of mMBP-specific Th1 cells by B10.BR APC.

As shown in Figures 10A-10B, depletion of CyCAP using either specific antiserum followed by protein A beads (A) or Cyp C-GST fusion protein bound to glutathione-agarose (B) significantly diminished the down-regulatory activity of the B10.A two-hour culture supernatant. $2.5 - 5 \times 10^5$ TCL-4 cells/ml were stimulated with 30 μM mMBP Ac 1-16 and $2.5 - 5 \times 10^5$ B10.BR peritoneal macrophages in either B10.A or B10.BR two-hour culture supernatants depleted of CyCAP by using anti-CyCAP specific antibody followed by protein A beads (A); or by using Cyp C-GST-glutathione beads (B) or in supernatant incubated with controls: Normal Rat Serum for the antibody-based depletion and PBS for the Cyp C-GST-based depletion. Results represent means and variances of duplicate wells of TNF-α_bioassay. Two total experiments each with the addition of antibody and Cyp C-GST depletion methods produced similar results.

The levels of TNF-α produced by MBP-specific TCL-4, stimulated with mMBP Ac 1-16 and B10.BR APC, increased and became similar to those produced in the presence of the B10.BR

two hour culture supernatant. The residual down-regulatory activity seen in panel B may represent residual CyCAP, as the amount of Cyp C-GST necessary for the maximal depletion was not determined.

To confirm the role of CyCAP in CYTRF activity, purified native B10.A CyCAP was added to the Th1 cells during the antigenic stimulation with syngeneic B10.BR peritoneal macrophages. Figures 11A-11D show that B10.A-derived CyCAP down-regulated TNF-α (A) and IFN-γ (B) levels produced by mMBP-specific TCL 4. 5 x 10^5 TCL-4 were stimulated with 30 μM mMBP Ac1-16 and 5 x 10^5 B10.BR macrophages with addition of serial dilutions of the B10.A-derived CyCAP purified from either peritoneal fluid or from culture supernatants from peritoneal macrophages cultured for 1 hour in vitro with no added stimuli. TNF-α (A) and IFN-γ (B) levels in the supernatants were determined after 24 hours, and the incorporation of ^{3}H-thymidine (C) was assayed after 48 hours, as above. Similar results were obtained in an additional experiment.

CyCAP did not diminish the proliferative response to mMBP peptide (Figure 11C), which is consistent with previous observed finding of similar proliferative responses of B10.BR TCL stimulated with either B10.A or B10.BR APC. The purity of the CyCAP was confirmed by the SDS-PAGE gel of the ^{35}S-metabolically labeled, macrophage secreted proteins, purified with the Cyp C-GST affinity beads and eluted with CsA. 1 x 10^6 thioglycollate-elicited B10.A peritoneal macrophages/ml were metabolically labeled with ^{35}S-methionine, and CyCAP purified with Cyp C-GST-coated glutathione beads and eluted with CsA was resolved on a 10% SDS-PAGE gel. Shown CyCAP purified from duplicate wells, as imaged by digital camera. The migration position of 97.4 kD marker protein is indicated at the right. Similarly, highly enriched CyCAP protein was identified by silver-stained SDS-PAGE gel. These results suggest that CYTRF activity is associated with CyCAP.

Effectiveness of CYTRF in down-regulating IFN-γ and TNF-α depends on the nature of signaling via TCR. The effects of CYTRF on IFN-γ and TNF-α production were tested in the MBP and Hb systems. Th1 cells were differentially stimulated by the wild type peptide or peptide analogues known to elicit different T cell responses. Figures 12A-12B show proliferative responses of TCL 4 (Panel A) and 3L2 (Panel B) to different antigens, reflecting the effects of the altered peptide ligands on the level of T cell stimulation. 2 x 10^4 T cells/100 μl were stimulated with serial dilutions of peptides presented by 1 x 10^5 B10.BR lethally-irradiated splenic APC in one well of 96 well plate. 48 hours later 1 μCi ^{3}H-thymidine was added to each well, and incorporation of the ^{3}H-thymidine was measured as described above. Similar results were obtained in at least two independent experiments.

The strong agonist peptide mMBP Ac 1-16(4E) induced proliferation of T cells at about 1 - 2 log lower concentration compared to the partial agonist mMBP Ac 1-16. Addition of the inhibitory

anti-CD4 antibody shifted the mMBP Ac 1-16(4E) dose-response toward that of the mMBP Ac 1-16. Similarly, the wt Hb peptide induced proliferative responses at significantly lower doses, compared to partial agonist Hb(T). The antagonist peptide Hb(I) did not induce proliferative response even at 100 μM. The proliferative responses of Hb-specific 3L2 cells correlated very well with the previously-determined induction of IL-2 production and of cytotoxic activity on a B cell line used as APC, the assay previously used to characterize agonistic/anagonistic properties and EC_{40} of the Hb analogues. The relative activities of T cell stimulation were determined as 100% for the wt Hb, 2% for Hb(T) and 0.006% for Hb(I); the EC_{40} was established to be 0.0004 μM for wt Hb, 0.023 μM for Hb(T), and 7 μM for Hb(I).

Figure 13A shows that when TCL 4 cells were stimulated with the wild type mMBP Ac1-16, CYTRF down-regulated TNF-α and IFN-γ_production; however, stimulation of Th1 cells with a stronger agonist, mMBP Ac1-16(4E), did not permit such down-regulation. 3×10^5 TCL-4 cells/ml were stimulated with 30 μM mMBP Ac1-16, 0.3 μM mMBP Ac1-16(4E), or 3 μM mMBP Ac1-16(4E) + 1 μg/ml anti-CD4 presented by 3×10^5 B10.A or B10.BR peritoneal macrophages. Figure 13B: $2x\ 10^5$ 3L2 cells/ml were stimulated with 10 μM wild type (wt) Hb 64-76 or 100 μM Hb(T), or 100 μM Hb(I) presented by 3×10^5 B10.A or B10.BR peritoneal macrophages. Culture supernatants were assayed for TNF-α and IFN-γ 24 hours later. Presented are means and standard deviations of triplicate wells of the TNF-α bioassay and IFN-γ ELISA; except for TNF-α levels in 6A, which represent means and variances of duplicate wells of the TNF-α bioassay. Similar results were obtained in at least three experiments.

Decreasing T cell stimulation by blocking CD4 signaling with anti-CD4 antibody again restored the inhibitory activity of the B10.A macrophages. Similar results were observed with B10.BR APC in the presence of B10.A vs. B10.BR two-hour culture supernatants. Adding the blocking anti-CD4 antibodies to TCL 4 stimulated by the wild type mMBP Ac1-16 resulted in a very low cytokine response, and did not increase, but at times even diminished differences in responses elicited by the B10.A and B10.BR APC.

Similarly, B10.A-derived down-regulatory effects were significantly lower when the strong agonist, the wild type 64-76 Hb peptide, was used in the stimulation of the 3L2 cells compared to the partial agonist Hb(T) (Figure 13B). Hb(T) allowed B10.A macrophages to robustly inhibit Th1 cytokine production. Under very low levels of T cell stimulation (Hb(I)) the difference between the B10.A and B10.BR macrophages in down-regulation of TNF-α was not observed; although IFN-γ production was inhibited, very low levels of IFN-γ were induced by the stimulation with APC from either strain. Stimulation of T cells with a combination of wt Hb and Hb(T), potentially allowing signaling by both peptide ligands, resulted in the diminished production of TNF-α and IFN-γ compared to that observed in the presence of wt Hb alone and again restored the down-regulatory activity of B10.A-derived CYTRF (Figures 14A-14B). 3×10^5 B10.A or B10.BR peritoneal

macrophages/ml were cultured with 10 μM wt Hb during adherence purification. After 1 hour macrophages were washed with cRPMI, and 3 x 10^5 3L2 T cells were added to these APC simultaneously with 100 μM Hb(T). As controls, 3L2 cells were stimulated by 10 μM wt Hb and 100 μM Hb(T) alone. 24 hours later culture supernatants were collected and assayed for TNF-α and IFN-γ levels.

These results suggest that: 1) Hb(T) displays antagonistic properties, and 2) signaling by a partial agonist/antagonist is dominant over that of a strong agonist in the induction of intracellular events that allow down-regulation of TNF-α and IFN-γ by B10.A-derived CYTRF. Similar results were observed when 100 μM mMBP Ac 1-16 was used in combination with 0.1 - 5 μM mMBP Ac1-16(4E). At lower than 100 μM concentrations mMBP Ac 1-16 could not restore the ability of B10.A-derived CYTRF to down-regulate Th1 cytokine production, probably because it could not productively compete with the mMBP Ac1-16(4E) for the binding to the IA^k.

The Th1 down-regulatory effects of purified CyCAP were also tested in Hb system. 10 nM CyCAP significantly inhibited the production of TNF-α and IFN-γ by the Hb-specific 3L2 cells (Figures 15A and 15B). 3 x 10^5 3L2 cells/ml were stimulated with 2 x 10^5 B10.BR peritoneal macrophages and either 10 μM wt Hb or 100 μM Hb (T) in the presence or in the absence of 10 nM CyCAP. TNF-α (A) and IFN-γ (B) were assayed 24 hours after stimulation. Serial dilutions of B10.A-derived CyCAP (C) were added to the similarly stimulated 3L2 cells, but 1 μM wt Hb and 10 μM Hb (T) were used. Shown are means and standard deviations or variances of triplicate wells for TNF-α bioassay and duplicate wells for the IFN-γ ELISA. Similar results were obtained in an additional experiment.

In surprising contrast with the results seen with either B10.A macrophages or with B10.A two-hour culture supernatant, the magnitude of inhibition by 10 nM highly purified CyCAP was at least as pronounced for the strong antigen, the wild type Hb, as for the Hb(T). However, the inhibitory effects of the B10.A-derived CyCAP were sustained at lower CyCAP concentrations when T cells were stimulated by the partial agonist Hb(T) (Figure 15C), suggesting that at higher than physiological levels CyCAP can down-regulate Th1 cytokine production induced by strong agonists. The effectiveness of the B10.A-derived down-regulatory activity depends on the strength or nature of T cell stimulation. The most prominent down-regulation is observed during sub-optimal levels of T cell activation. These data also support the involvement of CyCAP in the CYTRF-induced down-regulation of Th1 cytokines.

CyCAP mRNA levels in B10.A and B10.BR macrophages. RT PCR analysis of the CyCAP mRNA demonstrated that 1) there is no major difference in the levels of CyCAP mRNA between B10.A and B10.BR macrophages; 2) levels of CyCAP mRNA decline by 24 hours of culture; and 3) in addition to the CyCAP mRNA of the predicted 1902 bp size (12) other mRNA(s)

of lower molecular weight are consistently amplified. These results imply that the genetic difference between the B10.A and B10.BR in CYTRF activity does not reflect differences in the levels of CyCAP mRNA, and that CyCAP mRNA levels are either positively regulated *in vivo* or are negatively affected by some *in vitro* culture conditions. Multiple mRNA species could represent genes with regions homologous to the PCR primers, or alternatively spliced forms of CyCAP mRNA.

Production of IL-12 by B10.A, B10.BR, (B10.A x B10.BR)F1, CyCAP $^{+/+}$, and CyCAP $^{-/-}$ macrophages. As described above, CYTRF down-regulatory activity for IFN-γ and TNF-α was shown to be produced by macrophages. Serum levels of IFN-γ and TNF-α are elevated in CyCAP $^{-/-}$ mice following injections of LPS. As macrophages are known to produce IL-12, a major positive regulator of IFN-γ, IL-12 production by activated B10.A, B10.BR and (B10.A x B10.BR)F1 macrophages, and by macrophages from CyCAP $^{+/+}$ and CyCAP $^{-/-}$ mice has been compared.

B10.A, B10.BR, and (B10.A x B10.BR)F1 peritoneal macrophages elicited with thioglycollate were activated *in vitro* by LPS and IFN-γ. IFN-γ is known to augment LPS-induced IL-12 production in macrophages. Levels of IL-12 in the supernatants were tested 24 hours later. As shown in Figure 16A, IL-12 levels produced by B10.A macrophages were down-regulated by 60% compared to those produced by B10.BR macrophages. Figure 16A: adherence-purified thioglycollate-elicited peritoneal macrophages were cultured at 2.5-5 x 10^5/ml and stimulated with 20 μg/ml LPS and 100 U/ml IFN-γ. 24 hours later supernatants were harvested and tested for levels of IL-12 using IL-12 ELISA. Results represent means and standard errors of four independent experiments. Figure 16B: In experiment #1, adherence-purified resident peritoneal macrophages were cultured at 2.5 x 10^5/ml and stimulated with 10 μg/ml LPS and 100 U/ml IFN-γ. In experiment #2 adherence-purified thioglycollate-elicited macrophages were cultured at 4 x 10^5/ml and similarly stimulated. 9 hours later supernatants were harvested and tested for levels of IL-12 using IL-12 ELISA. Results represent means of duplicate wells with variances within 22%.

The lower IL-12 production was dominant in the (B10.A x B10.BR)F1 macrophages. Levels of TNF-α measured either at 2 hours or at 24 hours after the activation were not consistently different between the two B10 strains, with macrophages from each strain variably producing higher, lower, or similar levels of this cytokine. Variation in TNF-α production may reflect differential delivery of thioglycollate injections and/or differences in macrophage isolation/viability. Therefore, only when TNF-α levels were similar between the macrophages from two B10 strains were IL-12 levels compared.

Similar experiments were performed with macrophages from CyCAP $^{+/+}$ and CyCAP $^{-/-}$ mice. As shown in Figure 16B, CyCAP $^{-/-}$ macrophages produced higher levels of IL-12 compared to macrophages from CyCAP $^{+/+}$ strain. This general conclusion, however, was accompanied by a

large variation between experiments in total levels of IL-12 being produced and in the magnitude of differences between CyCAP $^{+/+}$ and CyCAP $^{-/-}$ mice. Macrophages used in experiment #1 were from mice derived from three backcrosses onto the C57/BL background and were not elicited with thioglycollate. However, IL-12 production by these resident cells was at least three fold higher compared to macrophages used in the experiment #2, derived from F3 mice of crosses onto the 129 background and elicited with thioglycollate, which usually results in higher macrophage activation. In a third experiment, normal resident macrophages were derived from CyCAP $^{+/+}$ and CyCAP $^{-/-}$ F3 mice on the 129 background. These cells produced only low levels of IL-12 and did not show a significant difference in IL-12 levels between CyCAP $^{+/+}$ and CyCAP $^{-/-}$ macrophages.

These results suggest that genetic difference between B10.A and B10.BR strains may control IL-12 production in response to LPS. Similarly to the effects of B10.A-derived CYTRF, CyCAP appears to be involved in the down-regulation of the IL-12 response, indicated by the enhanced IL-12 production when this gene is not functional.

Characterization of CYTRF activity has identified several physiologically important points. First, the inhibitory activity is made by macrophages. As these cells are efficient APC which, unlike B cells, generally promote Th1 cytokine responses, the differential genetic control of TNF-α and IFN-γ production by macrophages is very relevant. The capability of B10.A macrophages to inhibit the effector functions of Th1 cells, while not affecting IL-2 and IL-4 production, would limit the induction of DTH. The physiological significance of CYTRF is suggested by the studies connecting the effectiveness of the B10.A-derived inhibitory activity with the degree of T cell activation. Strong T cell activation signals induced by true agonists appear to make the cells resistant to the down-regulation of TNF-α and IFN-γ by CYTRF. However, inhibition of CD4 signaling or signaling by partial agonists/antagonists (alone or in combination with the strong agonist) permitted the down-regulation of these Th1 cytokines by the B10.A-derived factor. Thus, the genetically-determined difference between B10.A and B10.BR strains in Th1 cytokine production is manifested mainly when T cells are activated sub-optimally, reflecting the strength and/or nature of the activating stimuli.

This conclusion is relevant to the regulation of potential self-reactivity, as T cells strongly responding to self are eliminated and/or inactivated by several layers of negative control, but weakly self-reactive T cells may escape such negative regulation. Th1 cytokine production in response to weak self-antigens may suffice to induce an autoimmune disease. The activity of CYTRF allows inhibition of the Th1 effector responses to partial agonists, either self-peptides or pathogenic peptides, while allowing T cell proliferation and help for anti-pathogenic antibody production.

The present results suggest that susceptibility to CYTRF-mediated inhibition of the TNF-α and IFN-γ cytokine production is controlled by qualitatively and not by quantitatively different

signals through the TCR. The dosage of an antigen is not as important as its agonistic character, as TNF-α and IFN-γ were successfully down-regulated when a 10-fold range of concentrations of partial agonists were used, and similarly, resistance to the Th1 cytokine inhibition by CYTRF was observed in a 10-fold range of strong agonists. As signaling by agonists, partial agonists and antagonists corresponds to qualitatively-different patterns of the CD3 and ZAP-70 tyrosine phosphorylation, one can identify "permissive" vs. "inhibitory" signal transduction pathways down-stream of the TCR, leading to the characterization of the critical kinetic and/or molecular events in T cell activation determining whether TNF-α and IFN-γ will be produced at high levels or down-regulated. The B10.A-derived down-regulatory activity is dependent on CyCAP. Effects of depletion and addition of CyCAP on TNF-α and IFN-γ production, as well as the biochemical parameters support this conclusion. The addition of CyCAP does not affect T cell proliferation, which is also similar to the phenotypes of the B10.A-derived CYTRF. Finally, IL-12 levels produced by CyCAP knockout macrophages in response to LPS + IFN-γ were often higher than those produced by the CyCAP wild type cells, suggesting that CyCAP similarly to CYTRF negatively regulates production of IL-12.

Highly-purified B10.A-derived CyCAP down-regulated TNF-α and IFN-γ production by mMBP-specific and Hb-specific mature Th1 cells. The same preparation of CyCAP also significantly down-regulated IFN-γ levels produced by naive HEL-specific TCR transgenic T cells. As predicted, exogenous CyCAP did not induce IL-4 by the HEL TCR transgenic T cells. CYTRF exhibits a novel mechanism of genetic and molecular regulation of cytokine production and of the control of susceptibility/resistance to autoimmune diseases in mice and in humans.

All publications and patent applications cited in this specification are herein incorporated by reference as if each individual publication or patent application were specifically and individually indicated to be incorporated by reference.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be readily apparent to those of ordinary skill in the art in light of the teachings of this invention that certain changes and modifications may be made thereto without departing from the spirit or scope of the appended claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION

(i) APPLICANT: Conboy, Irina
Jones, Patricia

(ii) TITLE OF THE INVENTION: METHODS OF REGULATING T CELL AND MACROPHAGE CYTOKINE PRODUCTION, FUNCTION AND PATHOGENICITY

(iii) NUMBER OF SEQUENCES: 6

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: Bozicevic & Reed, LLP
(B) STREET: 285 Hamilton Avenue, Suite 200
(C) CITY: Palo Alto
(D) STATE: CA
(E) COUNTRY: USA
(F) ZIP: 94301

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Diskette
(B) COMPUTER: IBM Compatible
(C) OPERATING SYSTEM: DOS
(D) SOFTWARE: FastSEQ for Windows Version 2.0

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: 60/049,578
(B) FILING DATE: 13-JUN-1997

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Sherwood, Pamela J
(B) REGISTRATION NUMBER: 36,677

(C) REFERENCE/DOCKET NUMBER:

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: 650-327-3400
(B) TELEFAX: 650 327-3231
(C) TELEX:

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 2176 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: cDNA
(ix) FEATURE:

(A) NAME/KEY: Coding Sequence
(B) LOCATION: 176...1897
(D) OTHER INFORMATION: Mouse CYTRF cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

```
 CAGCCTCGTC CCAGGAAAGC CACATGGTGG AGCTTTTCTC TGCTGAACAG TCTACAGAAG
60
 GCTTCCAGTT GCAGCCAGGA AGGAGGCTCC AGGACTGCCT TCAGCCACTG CCTCGCTGCC
120
 TGAGGGACAG TTGAGTCCCT CTCTTGCTCC CAGGGTTGGG CTTCTAGGCC AGGCA ATG    178
                                                               Met
                                                                1

 GCT CTC CTG TGG CTC CTC TCT GTG TTC TTG CTG GTT CCA GGG ACT CAA     226
 Ala Leu Leu Trp Leu Leu Ser Val Phe Leu Leu Val Pro Gly Thr Gln
              5                  10                  15

 GGT ACA GAA GAT GGA GAC ATG GGC TTG GTT AAC GGG GCC TCA GCC AAT     274
 Gly Thr Glu Asp Gly Asp Met Gly Leu Val Asn Gly Ala Ser Ala Asn
         20                  25                  30

 GAG GGC CGC GTG GAG ATC TTC TAC AGA GGC CGG TGG GGG ACA GTG TGT     322
 Glu Gly Arg Val Glu Ile Phe Tyr Arg Gly Arg Trp Gly Thr Val Cys
     35                  40                  45
```

```
GAC AAC CTC TGG AAC CTT TTG GAT GCC CAC GTC GTC TGC CGG GCC CTG      370
Asp Asn Leu Trp Asn Leu Leu Asp Ala His Val Val Cys Arg Ala Leu
50                  55                  60                  65

GGC TAT GAG AAC GCC ACC CAA GCA CTG GGC AGA GCT GCC TTC GGG CCA      418
Gly Tyr Glu Asn Ala Thr Gln Ala Leu Gly Arg Ala Ala Phe Gly Pro
                70                  75                  80

GGA AAG GGA CCG ATC ATG CTG GAT GAG GTG GAA TGT ACA GGG ACC GAG      466
Gly Lys Gly Pro Ile Met Leu Asp Glu Val Glu Cys Thr Gly Thr Glu
            85                  90                  95

TCC TCA CTG GCC AGT TGC AGA TCC CTG GGT TGG ATG GTG AGC CGC TGT      514
Ser Ser Leu Ala Ser Cys Arg Ser Leu Gly Trp Met Val Ser Arg Cys
        100                 105                 110

GGG CAC GAG AAG GAC GCA GGC GTG GTC TGC TCC AAC GAT ACC ACG GGG      562
Gly His Glu Lys Asp Ala Gly Val Val Cys Ser Asn Asp Thr Thr Gly
    115                 120                 125

CTT CAC ATC CTG GAC CTC TCT GGA GAG CTC TCA GAT GCA CTG GGC CAG      610
Leu His Ile Leu Asp Leu Ser Gly Glu Leu Ser Asp Ala Leu Gly Gln
130                 135                 140                 145

ATC TTT GAC AGC CAG CAG GGC TGC GAC CTA TTC ATC CAG GTG ACA GGG      658
Ile Phe Asp Ser Gln Gln Gly Cys Asp Leu Phe Ile Gln Val Thr Gly
                150                 155                 160

CAG GGG TAT GAG GAC CTG AGC CTC TGT GCC CAC ACG CTG ATC CTG CGC      706
Gln Gly Tyr Glu Asp Leu Ser Leu Cys Ala His Thr Leu Ile Leu Arg
            165                 170                 175

ACC AAC CCC GAG GCC CAG GCC CTG TGG CAA GTG GTG GGC AGC AGC GTC      754
Thr Asn Pro Glu Ala Gln Ala Leu Trp Gln Val Val Gly Ser Ser Val
        180                 185                 190

ATC ATG AGA GTG GAT GCT GAG TGC ATG CCT GTC GTC AGA GAC TTC CTC      802
Ile Met Arg Val Asp Ala Glu Cys Met Pro Val Val Arg Asp Phe Leu
    195                 200                 205

AGG TAC TTT TAC TCC CGA AGA ATC GAG GTC AGC ATG TCT TCT GTT AAG      850
Arg Tyr Phe Tyr Ser Arg Arg Ile Glu Val Ser Met Ser Ser Val Lys
210                 215                 220                 225
```

```
TGC TTG CCA AAG CTA GCC TCT GCC TAT GGG GCC ACA GAG CTT CAG GAC     898
Cys Leu Pro Lys Leu Ala Ser Ala Tyr Gly Ala Thr Glu Leu Gln Asp
                230                 235                 240

TAC TGT GGA CGG CTT TTT GCC ACC CTC CTC CCC CAA GAC CCC ACT TTC     946
Tyr Cys Gly Arg Leu Phe Ala Thr Leu Leu Pro Gln Asp Pro Thr Phe
            245                 250                 255

CAT ACT CCC TTG GAC CTT TAT GCG TAC GCA CGG GCC ACC GGG GAC TCT     994
His Thr Pro Leu Asp Leu Tyr Ala Tyr Ala Arg Ala Thr Gly Asp Ser
        260                 265                 270

ATG CTG GAA GAT CTG TGT GTA CAG TTT CTG GCC TGG AAC TTC GAG CCT    1042
Met Leu Glu Asp Leu Cys Val Gln Phe Leu Ala Trp Asn Phe Glu Pro
    275                 280                 285

CTG ACA CAG TCT GAG TCC TGG TCG GCT GTT CCC ACC ACC TTG ATC CAG    1090
Leu Thr Gln Ser Glu Ser Trp Ser Ala Val Pro Thr Thr Leu Ile Gln
290                 295                 300                 305

GCT CTC CTC CCC AAG AGT GAG CTG GCT GTG TCT AGT GAG CTG GAT CTG    1138
Ala Leu Leu Pro Lys Ser Glu Leu Ala Val Ser Ser Glu Leu Asp Leu
                310                 315                 320

CTG AAG GCA GTG GAC CAG TGG AGC ACA GAA ACC ATT GCC TCA CAC GAG    1186
Leu Lys Ala Val Asp Gln Trp Ser Thr Glu Thr Ile Ala Ser His Glu
            325                 330                 335

GAT ATA GAG CGC CTG GTG GAA CAG GTC CGC TTC CCC ATG ATG CTG CCC    1234
Asp Ile Glu Arg Leu Val Glu Gln Val Arg Phe Pro Met Met Leu Pro
        340                 345                 350

CAG GAG CTG TTC GAG CTG CAG TTC AAC CTG TCC TTG TAC CAA GAT CAC    1282
Gln Glu Leu Phe Glu Leu Gln Phe Asn Leu Ser Leu Tyr Gln Asp His
    355                 360                 365

CAG GCA CTG TTC CAG AGG AAG ACC ATG CAG GCC TTG GAG TTC CAC ACA    1330
Gln Ala Leu Phe Gln Arg Lys Thr Met Gln Ala Leu Glu Phe His Thr
370                 375                 380                 385

GTG CCT GTC GAA GTG CTG GCC AAG TAC AAA GGC CTG AAC CTC ACG GAG    1378
Val Pro Val Glu Val Leu Ala Lys Tyr Lys Gly Leu Asn Leu Thr Glu
                390                 395                 400
```

```
GAC ACC TAC AAG CCC CGC CTT TAC ACC TCT TCC ACC TGG AGT AGC TTG    1426
Asp Thr Tyr Lys Pro Arg Leu Tyr Thr Ser Ser Thr Trp Ser Ser Leu
            405                 410                 415

GTG ATG GCC TCC ACC TGG AGG GCA CAA AGA TAT GAA TAC AAT CGA TAC    1474
Val Met Ala Ser Thr Trp Arg Ala Gln Arg Tyr Glu Tyr Asn Arg Tyr
        420                 425                 430

AAT CAG CTC TAT ACA TAT GGC TAT GGC TCA GTA GCC CGG TAC AAT AGC    1522
Asn Gln Leu Tyr Thr Tyr Gly Tyr Gly Ser Val Ala Arg Tyr Asn Ser
    435                 440                 445

TAC CAG TCC TTC CAA ACC CCA CAA CAC CCC AGC TTC CTC TTC AAG GAC    1570
Tyr Gln Ser Phe Gln Thr Pro Gln His Pro Ser Phe Leu Phe Lys Asp
450                 455                 460                 465

AAC GAG ATC TCC TGG TCA GCC ACC TAC CTC CCC ACC ATG CAG AGC TGC    1618
Asn Glu Ile Ser Trp Ser Ala Thr Tyr Leu Pro Thr Met Gln Ser Cys
                470                 475                 480

TGG AAC TAT GGC TTC TCG TGT ACC TCT AAC GAG CTC CCT GTA CTG GGC    1666
Trp Asn Tyr Gly Phe Ser Cys Thr Ser Asn Glu Leu Pro Val Leu Gly
            485                 490                 495

CTC ACC ACA TCC AGC TAC TCC AAT CCG ACA ATT GGC TAT GAG AAC AGA    1714
Leu Thr Thr Ser Ser Tyr Ser Asn Pro Thr Ile Gly Tyr Glu Asn Arg
        500                 505                 510

GTA CTG ATC CTC TGC GGA GGC TAC AGT GTG GTG GAT GTC ACC AGC TTT    1762
Val Leu Ile Leu Cys Gly Gly Tyr Ser Val Val Asp Val Thr Ser Phe
    515                 520                 525

GAA GGC TCC AAG GCC CCT ATT CCC ACT GCC CTG GAC ACC AAT AGT TCC    1810
Glu Gly Ser Lys Ala Pro Ile Pro Thr Ala Leu Asp Thr Asn Ser Ser
530                 535                 540                 545

AAG ACT CCC TCC CTC TTT CCC TGT GCC TCA GGG GCC TTT AGC AGC TTC    1858
Lys Thr Pro Ser Leu Phe Pro Cys Ala Ser Gly Ala Phe Ser Ser Phe
                550                 555                 560

CGT GTG GTC ATA CGC CCC TTC TAC CTC ACT AAC CTC CAC TGACATGGTG TA  1909
Arg Val Val Ile Arg Pro Phe Tyr Leu Thr Asn Leu His
            565                 570
```

AATGGTACAT CTCAGTGGTG GGGACGCAGA CATTCCTGTG TCCCCTCCTT CGCCTCCAGC
1969
TGCTTTGTAA GCATAAACTG ATTGTATTCA AATGAAAATT TACTAGAAGG TTTCAGCCAG
2029
CACTCACTCC AGGACTGAGA GTCCCAGGGC CCTCACTGCA GGTACAAGCA GGTTCCATGA
2089
GGTCCTGTGG GATTTCCTGT GCCTACTGCA GTAGCCCCAT CTGTCACAGT CACTCATCAA
2149
AAATCATTAA AGTCTCACGT GCTTCTC
2176

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 574 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: protein
(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Met Ala Leu Leu Trp Leu Leu Ser Val Phe Leu Leu Val Pro Gly Thr
1 5 10 15
Gln Gly Thr Glu Asp Gly Asp Met Gly Leu Val Asn Gly Ala Ser Ala
20 25 30
Asn Glu Gly Arg Val Glu Ile Phe Tyr Arg Gly Arg Trp Gly Thr Val
35 40 45
Cys Asp Asn Leu Trp Asn Leu Leu Asp Ala His Val Val Cys Arg Ala
50 55 60
Leu Gly Tyr Glu Asn Ala Thr Gln Ala Leu Gly Arg Ala Ala Phe Gly
65 70 75 80
Pro Gly Lys Gly Pro Ile Met Leu Asp Glu Val Glu Cys Thr Gly Thr
85 90 95
Glu Ser Ser Leu Ala Ser Cys Arg Ser Leu Gly Trp Met Val Ser Arg
100 105 110
Cys Gly His Glu Lys Asp Ala Gly Val Val Cys Ser Asn Asp Thr Thr
115 120 125
Gly Leu His Ile Leu Asp Leu Ser Gly Glu Leu Ser Asp Ala Leu Gly
130 135 140
Gln Ile Phe Asp Ser Gln Gln Gly Cys Asp Leu Phe Ile Gln Val Thr
145 150 155 160
Gly Gln Gly Tyr Glu Asp Leu Ser Leu Cys Ala His Thr Leu Ile Leu
165 170 175

```
Arg Thr Asn Pro Glu Ala Gln Ala Leu Trp Gln Val Val Gly Ser Ser
            180                 185                 190
Val Ile Met Arg Val Asp Ala Glu Cys Met Pro Val Val Arg Asp Phe
        195                 200                 205
Leu Arg Tyr Phe Tyr Ser Arg Arg Ile Glu Val Ser Met Ser Ser Val
    210                 215                 220
Lys Cys Leu Pro Lys Leu Ala Ser Ala Tyr Gly Ala Thr Glu Leu Gln
225                 230                 235                 240
Asp Tyr Cys Gly Arg Leu Phe Ala Thr Leu Leu Pro Gln Asp Pro Thr
                245                 250                 255
Phe His Thr Pro Leu Asp Leu Tyr Ala Tyr Ala Arg Ala Thr Gly Asp
            260                 265                 270
Ser Met Leu Glu Asp Leu Cys Val Gln Phe Leu Ala Trp Asn Phe Glu
        275                 280                 285
Pro Leu Thr Gln Ser Glu Ser Trp Ser Ala Val Pro Thr Thr Leu Ile
    290                 295                 300
Gln Ala Leu Leu Pro Lys Ser Glu Leu Ala Val Ser Ser Glu Leu Asp
305                 310                 315                 320
Leu Leu Lys Ala Val Asp Gln Trp Ser Thr Glu Thr Ile Ala Ser His
                325                 330                 335
Glu Asp Ile Glu Arg Leu Val Glu Gln Val Arg Phe Pro Met Met Leu
            340                 345                 350
Pro Gln Glu Leu Phe Glu Leu Gln Phe Asn Leu Ser Leu Tyr Gln Asp
        355                 360                 365
His Gln Ala Leu Phe Gln Arg Lys Thr Met Gln Ala Leu Glu Phe His
    370                 375                 380
Thr Val Pro Val Glu Val Leu Ala Lys Tyr Lys Gly Leu Asn Leu Thr
385                 390                 395                 400
Glu Asp Thr Tyr Lys Pro Arg Leu Tyr Thr Ser Ser Thr Trp Ser Ser
                405                 410                 415
Leu Val Met Ala Ser Thr Trp Arg Ala Gln Arg Tyr Glu Tyr Asn Arg
            420                 425                 430
Tyr Asn Gln Leu Tyr Thr Tyr Gly Tyr Gly Ser Val Ala Arg Tyr Asn
        435                 440                 445
Ser Tyr Gln Ser Phe Gln Thr Pro Gln His Pro Ser Phe Leu Phe Lys
    450                 455                 460
Asp Asn Glu Ile Ser Trp Ser Ala Thr Tyr Leu Pro Thr Met Gln Ser
465                 470                 475                 480
Cys Trp Asn Tyr Gly Phe Ser Cys Thr Ser Asn Glu Leu Pro Val Leu
                485                 490                 495
Gly Leu Thr Thr Ser Ser Tyr Ser Asn Pro Thr Ile Gly Tyr Glu Asn
            500                 505                 510
Arg Val Leu Ile Leu Cys Gly Gly Tyr Ser Val Val Asp Val Thr Ser
        515                 520                 525
```

```
Phe Glu Gly Ser Lys Ala Pro Ile Pro Thr Ala Leu Asp Thr Asn Ser
    530                 535                 540
Ser Lys Thr Pro Ser Leu Phe Pro Cys Ala Ser Gly Ala Phe Ser Ser
545                 550                 555                 560
Phe Arg Val Val Ile Arg Pro Phe Tyr Leu Thr Asn Leu His
                565                 570
```

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 2257 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: cDNA
(ix) FEATURE:

(A) NAME/KEY: Coding Sequence
(B) LOCATION: 180...1934
(D) OTHER INFORMATION: Human CYTRF cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

```
 AATCGAAAGT AGACTCTTTT CTGAAGCATT TCCTGGGATC AGCCTGACCA CGCTCCATAC
60
 TGGGAGAGGC TTCTGGGTCA AAGGACCAGT CTGCAGAGGG ATCCTGTGGC TGGAAGCGAG
120
 GAGGCTCCAC ACGGCCGTTG CAGCTACCGC AGCCAGGATC TGGGCATCCA GGCACGGCC A
180
                                                                  Me

 TGA CCC CTC CGA GGC TCT TCT GGG TGT GGC TGC TGG TTG CAG GAA CCC      228
 t Thr Pro Pro Arg Leu Phe Trp Val Trp Leu Leu Val Ala Gly Thr Gl
1               5                  10                  15

 AAG GCG TGA ATG ATG GTG ACA TGC GGC TGG CCG ATG GGG GCG CCA CCA      276
 n Gly Val Asn Asp Gly Asp Met Arg Leu Ala Asp Gly Gly Ala Thr As
          20                  25                  30

 ACC AGG GCC GCG TGG AGA TCT TCT ACA GAG GCC AGT GGG GCA CTG TGT      324
 n Gln Gly Arg Val Glu Ile Phe Tyr Arg Gly Gln Trp Gly Thr Val Cy
      35                  40                  45
```

```
GTG ACA ACC TGT GGG ACC TGA CTG ATG CCA GCG TCG TCT GCC GGG CCC      372
s Asp Asn Leu Trp Asp Leu Thr Asp Ala Ser Val Val Cys Arg Ala Le
  50                  55                  60                  6

TGG GCT TCG AGA ACG CCA CCC AGG CTC TGG GCA GAG CTG CCT TCG GGC      420
u Gly Phe Glu Asn Ala Thr Gln Ala Leu Gly Arg Ala Ala Phe Gly Gl
5                 70                  75                  80

AAG GAT CAG GCC CCA TCA TGC TGG ACG AGG TCC AGT GCA CGG GAA CCG      468
n Gly Ser Gly Pro Ile Met Leu Asp Glu Val Gln Cys Thr Gly Thr Gl
              85                  90                  95

AGG CCT CAC TGG CCG ACT GCA AGT CCC TGG GCT GGC TGA AGA GCA ACT      516
u Ala Ser Leu Ala Asp Cys Lys Ser Leu Gly Trp Leu Lys Ser Asn Cy
          100                 105                 110

GCA GGC ACG AGA GAG ACG CTG GTG TGG TCT GCA CCA ATG AAA CCA GGA      564
s Arg His Glu Arg Asp Ala Gly Val Val Cys Thr Asn Glu Thr Arg Se
      115                 120                 125

GCA CCC ACA CCC TGG ACC TCT CCA GGG AGC TCT CGG AGG CCC TTG GCC      612
r Thr His Thr Leu Asp Leu Ser Arg Glu Leu Ser Glu Ala Leu Gly Gl
  130                 135                 140                 1

AGA TCT TTG ACA GCC AGC GGG GCT GCG ACC TGT CCA TCA GCG TGA ATG      660
n Ile Phe Asp Ser Gln Arg Gly Cys Asp Leu Ser Ile Ser Val Asn Va
45                150                 155                 160

TGC AGG GCG AGG ACG CCC TGG GCT TCT GTG GCC ACA CGG TCA TCC TGA      708
l Gln Gly Glu Asp Ala Leu Gly Phe Cys Gly His Thr Val Ile Leu Th
              165                 170                 175

CTG CCA ACC TGG AGG CCC AGG CCC TGT GGA AGG AGC CGG GCA GCA ATG      756
r Ala Asn Leu Glu Ala Gln Ala Leu Trp Lys Glu Pro Gly Ser Asn Va
          180                 185                 190

TCA CCA TGA GTG TGG ATG CTG AGT GTG TGC CCA TGG TCA GGG ACC TTC      804
l Thr Met Ser Val Asp Ala Glu Cys Val Pro Met Val Arg Asp Leu Le
      195                 200                 205

TCA GGT ACT TCT ACT CCC GAA GGA TTG ACA TCA CCC TGT CGT CAG TCA      852
u Arg Tyr Phe Tyr Ser Arg Arg Ile Asp Ile Thr Leu Ser Ser Val Ly
  210                 215                 220                 2
```

AGT GCT TCC ACA AGC TGG CCT CTG CCT ATG GGG CCA GGC AGC TGC AGG 900
s Cys Phe His Lys Leu Ala Ser Ala Tyr Gly Ala Arg Gln Leu Gln Gl
25 230 235 240

GCT ACT GCG CAA GCC TCT TTG CCA TCC TCC TCC CCC AGG ACC CCT CGT 948
y Tyr Cys Ala Ser Leu Phe Ala Ile Leu Leu Pro Gln Asp Pro Ser Ph
245 250 255

TCC AGA TGC CCC TGG ACC TGT ATG CCT ATG CAG TGG CCA CAG GGG ACG 996
e Gln Met Pro Leu Asp Leu Tyr Ala Tyr Ala Val Ala Thr Gly Asp Al
260 265 270

CCC TGC TGG AGA AGC TCT GCC TAC AGT TCC TGG CCT GGA ACT TCG AGG 1044
a Leu Leu Glu Lys Leu Cys Leu Gln Phe Leu Ala Trp Asn Phe Glu Al
275 280 285

CCT TGA CGC AGG CCG AGG CCT GGC CCA GTG TCC CCA CAG ACC TGC TCC 1092
a Leu Thr Gln Ala Glu Ala Trp Pro Ser Val Pro Thr Asp Leu Leu Gl
290 295 300 3

AAC TGC TGC TGC CCA GGA GCG ACC TGG CGG TGC CCA GCG AGC TGG CCC 1140
n Leu Leu Leu Pro Arg Ser Asp Leu Ala Val Pro Ser Glu Leu Ala Le
05 310 315 320

TAC TGA AGG CCG TGG ACA CCT GGA GCT GGG GGG AGC GTG CCT CCC ATG 1188
u Leu Lys Ala Val Asp Thr Trp Ser Trp Gly Glu Arg Ala Ser His Gl
325 330 335

AGG AGG TGG AGG GCT TGG TGG AGA AGA TCC GCT TCC CCA TGA TGC TCC 1236
u Glu Val Glu Gly Leu Val Glu Lys Ile Arg Phe Pro Met Met Leu Pr
340 345 350

CTG AGG AGC TCT TTG AGC TGC AGT TCA ACC TGT CCC TGT ACT GGA GCC 1284
o Glu Glu Leu Phe Glu Leu Gln Phe Asn Leu Ser Leu Tyr Trp Ser Hi
355 360 365

ACG AGG CCC TGT TCC AGA AGA AGA CTC TGC AGG CCC TGG AAT TCC ACA 1332
s Glu Ala Leu Phe Gln Lys Lys Thr Leu Gln Ala Leu Glu Phe His Th
370 375 380 3

CTG TGC CCT TCC AGT TGC TGG CCC GGT ACA AAG GCC TGA ACC TCA CCG 1380
r Val Pro Phe Gln Leu Leu Ala Arg Tyr Lys Gly Leu Asn Leu Thr Gl
85 390 395 400

```
AGG ATA CCT ACA AGC CCC GGA TTT ACA CCT CGC CCA CCT GGA GTG CCT   1428
u Asp Thr Tyr Lys Pro Arg Ile Tyr Thr Ser Pro Thr Trp Ser Ala Ph
              405                 410                 415

TTG TGA CAG ACA GTT CCT GGA GTG CAC GGA AGT CAC AAC TGG TCT ATC   1476
e Val Thr Asp Ser Ser Trp Ser Ala Arg Lys Ser Gln Leu Val Tyr Gl
          420                 425                 430

AGT CCA GAC GGG GGC CTT TGG TCA AAT ATT CTT CTG ATT ACT TCC AAG   1524
n Ser Arg Arg Gly Pro Leu Val Lys Tyr Ser Ser Asp Tyr Phe Gln Al
      435                 440                 445

CCC CCT CTG ACT ACA GAT ACT ACC CCT ACC AGT CCT TCC AGA CTC CAC   1572
a Pro Ser Asp Tyr Arg Tyr Tyr Pro Tyr Gln Ser Phe Gln Thr Pro Gl
  450                 455                 460                 4

AAC ACC CCA GCT TCC TCT TCC AGG ACA AGA GGG TGT CCT GGT CCC TGG   1620
n His Pro Ser Phe Leu Phe Gln Asp Lys Arg Val Ser Trp Ser Leu Va
65                  470                 475                 480

TCT ACC TCC CCA CCA TCC AGA GCT GCT GGA ACT ACG GCT TCT CCT GCT   1668
l Tyr Leu Pro Thr Ile Gln Ser Cys Trp Asn Tyr Gly Phe Ser Cys Se
              485                 490                 495

CCT CGG ACG AGC TCC CTG TCC TGG GCC TCA CCA AGT CTG GCG GCT CAG   1716
r Ser Asp Glu Leu Pro Val Leu Gly Leu Thr Lys Ser Gly Gly Ser As
          500                 505                 510

ATC GCA CCA TTG CCT ACG AAA ACA AAG CCC TGA TGC TCT GCG AAG GGC   1764
p Arg Thr Ile Ala Tyr Glu Asn Lys Ala Leu Met Leu Cys Glu Gly Le
      515                 520                 525

TCT TCG TGG CAG ACG TCA CCG ATT TCG AGG GCT GGA AGG CTG CGA TTC   1812
u Phe Val Ala Asp Val Thr Asp Phe Glu Gly Trp Lys Ala Ala Ile Pr
  530                 535                 540                 5

CCA GTG CCC TGG ACA CCA ACA GCT CGA AGA GCA CCT CCT CCT TCC CCT   1860
o Ser Ala Leu Asp Thr Asn Ser Ser Lys Ser Thr Ser Ser Phe Pro Cy
45                  550                 555                 560

GCC CGG CAG GGC ACT TCA ACG GCT TCC GCA CGG TCA TCC GCC CCT TCT   1908
s Pro Ala Gly His Phe Asn Gly Phe Arg Thr Val Ile Arg Pro Phe Ty
              565                 570                 575
```

ACC TGA CCA ACT CCT CAG GTG TGG AC TAGACGGCGT GGCCCAAGGG TGGTGAGA 1962
r Leu Thr Asn Ser Ser Gly Val Asp
580 585

ACCGGAGAAC CCCAGGACGC CCTCACTGCA GGCTCCCCTC CTCGGCTTCC TTCCTCTCTG
2022
CAATGACCTT CAACAACCGG CCACCAGATG TCGCCCTACT CACCTGAGCG CTCAGCTTCA
2082
AGAAATTACT GGAAGGCTTC CACTAGGGTC CACCAGGAGT TCTCCCACCA CCTCACCAGT
2142
TTCCAGGTGG TAAGCACCAG GACGCCCTCG AGGTTGCTCT GGGATCCCCC CACAGCCCCT
2202
GGTCAGTCTG CCCTTGTCAC TGGTCTGAGG TCATTAAAAT TACATTGAGG TTCCT
2257

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 585 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: protein
(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Met Thr Pro Pro Arg Leu Phe Trp Val Trp Leu Leu Val Ala Gly Thr
1 5 10 15
Gln Gly Val Asn Asp Gly Asp Met Arg Leu Ala Asp Gly Gly Ala Thr
20 25 30
Asn Gln Gly Arg Val Glu Ile Phe Tyr Arg Gly Gln Trp Gly Thr Val
35 40 45
Cys Asp Asn Leu Trp Asp Leu Thr Asp Ala Ser Val Val Cys Arg Ala
50 55 60
Leu Gly Phe Glu Asn Ala Thr Gln Ala Leu Gly Arg Ala Ala Phe Gly
65 70 75 80
Gln Gly Ser Gly Pro Ile Met Leu Asp Glu Val Gln Cys Thr Gly Thr
85 90 95
Glu Ala Ser Leu Ala Asp Cys Lys Ser Leu Gly Trp Leu Lys Ser Asn
100 105 110
Cys Arg His Glu Arg Asp Ala Gly Val Val Cys Thr Asn Glu Thr Arg
115 120 125
Ser Thr His Thr Leu Asp Leu Ser Arg Glu Leu Ser Glu Ala Leu Gly
130 135 140

```
Gln Ile Phe Asp Ser Gln Arg Gly Cys Asp Leu Ser Ile Ser Val Asn
145                 150                 155                 160
Val Gln Gly Glu Asp Ala Leu Gly Phe Cys Gly His Thr Val Ile Leu
                165                 170                 175
Thr Ala Asn Leu Glu Ala Gln Ala Leu Trp Lys Glu Pro Gly Ser Asn
            180                 185                 190
Val Thr Met Ser Val Asp Ala Glu Cys Val Pro Met Val Arg Asp Leu
        195                 200                 205
Leu Arg Tyr Phe Tyr Ser Arg Arg Ile Asp Ile Thr Leu Ser Ser Val
    210                 215                 220
Lys Cys Phe His Lys Leu Ala Ser Ala Tyr Gly Ala Arg Gln Leu Gln
225                 230                 235                 240
Gly Tyr Cys Ala Ser Leu Phe Ala Ile Leu Leu Pro Gln Asp Pro Ser
                245                 250                 255
Phe Gln Met Pro Leu Asp Leu Tyr Ala Tyr Ala Val Ala Thr Gly Asp
            260                 265                 270
Ala Leu Leu Glu Lys Leu Cys Leu Gln Phe Leu Ala Trp Asn Phe Glu
        275                 280                 285
Ala Leu Thr Gln Ala Glu Ala Trp Pro Ser Val Pro Thr Asp Leu Leu
    290                 295                 300
Gln Leu Leu Leu Pro Arg Ser Asp Leu Ala Val Pro Ser Glu Leu Ala
305                 310                 315                 320
Leu Leu Lys Ala Val Asp Thr Trp Ser Trp Gly Glu Arg Ala Ser His
                325                 330                 335
Glu Glu Val Glu Gly Leu Val Glu Lys Ile Arg Phe Pro Met Met Leu
            340                 345                 350
Pro Glu Glu Leu Phe Glu Leu Gln Phe Asn Leu Ser Leu Tyr Trp Ser
        355                 360                 365
His Glu Ala Leu Phe Gln Lys Lys Thr Leu Gln Ala Leu Glu Phe His
    370                 375                 380
Thr Val Pro Phe Gln Leu Leu Ala Arg Tyr Lys Gly Leu Asn Leu Thr
385                 390                 395                 400
Glu Asp Thr Tyr Lys Pro Arg Ile Tyr Thr Ser Pro Thr Trp Ser Ala
                405                 410                 415
Phe Val Thr Asp Ser Ser Trp Ser Ala Arg Lys Ser Gln Leu Val Tyr
            420                 425                 430
Gln Ser Arg Arg Gly Pro Leu Val Lys Tyr Ser Ser Asp Tyr Phe Gln
        435                 440                 445
Ala Pro Ser Asp Tyr Arg Tyr Tyr Pro Tyr Gln Ser Phe Gln Thr Pro
    450                 455                 460
Gln His Pro Ser Phe Leu Phe Gln Asp Lys Arg Val Ser Trp Ser Leu
465                 470                 475                 480
Val Tyr Leu Pro Thr Ile Gln Ser Cys Trp Asn Tyr Gly Phe Ser Cys
                485                 490                 495
```

```
Ser Ser Asp Glu Leu Pro Val Leu Gly Leu Thr Lys Ser Gly Gly Ser
            500                 505                 510
Asp Arg Thr Ile Ala Tyr Glu Asn Lys Ala Leu Met Leu Cys Glu Gly
        515                 520                 525
Leu Phe Val Ala Asp Val Thr Asp Phe Glu Gly Trp Lys Ala Ala Ile
    530                 535                 540
Pro Ser Ala Leu Asp Thr Asn Ser Ser Lys Ser Thr Ser Ser Phe Pro
545                 550                 555                 560
Cys Pro Ala Gly His Phe Asn Gly Phe Arg Thr Val Ile Arg Pro Phe
                565                 570                 575
Tyr Leu Thr Asn Ser Ser Gly Val Asp
            580                 585
```

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: Other

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

ATGGCTCTCC TGTGGCTCCT CTC 23

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: Other

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

CACCATGTCA GTGGAGTTAG TGAG 24

WHAT IS CLAIMED IS:

1. A method for decreasing the synthesis of pro-inflammatory cytokines in a T cell population, the method comprising:

contacting said T cell population with a formulation comprising CYTRF as an active agent for a period of time sufficient to reduce the synthesis of pro-inflammatory cytokines.

2. A method according to Claim 1, wherein said pro-inflammatory cytokines comprise γ-interferon and tumor necrosis factor α.

3. A method according to Claim 1, wherein said T cell population is synthesizing said pro-inflammatory cytokines in response to a weak antigen.

4. A method according to Claim 3, wherein said weak antigen is an autoantigen.

5. A method according to Claim 4, wherein said autoantigen is associated with autoimmune disease.

6. A method according to Claim 1, wherein said contacting comprises administration *in vivo* to an infected host.

7. A method according to Claim 1, wherein said contacting comprises administration *in vitro*.

8. A method according to Claim 1, wherein said CYTRF is human CYTRF .

9. A method according to Claim 8, wherein said human CYTRF comprises the amino acid sequence as set forth in SEQ ID NO:4.

10. A method according to Claim 8, wherein said human CYTRF comprises an amino acid sequence substantially similar to that set forth in SEQ ID NO:4.

11. An anti-inflammatory pharmaceutical composition, comprising:

CYTRF in an amount effective to reduce the synthesis of pro-inflammatory cytokines by a T cell population; and

a pharmaceutically acceptable carrier.

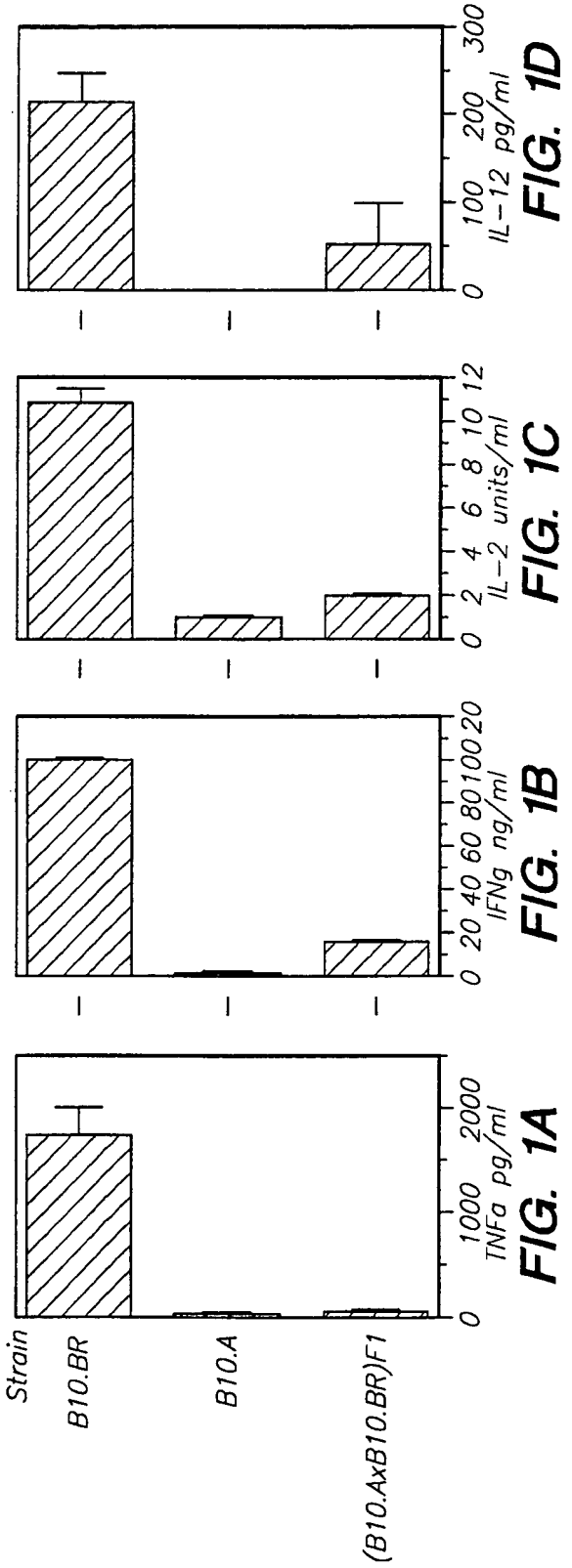


FIG. 1A

FIG. 1B

FIG. 1C

FIG. 1D

FIG. 1E

FIG. 1F

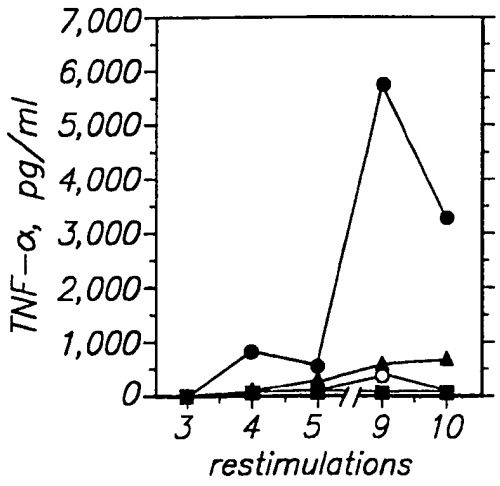


FIG. 2A

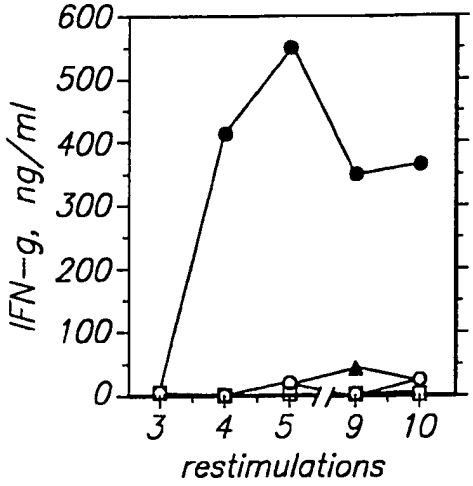


FIG. 2B

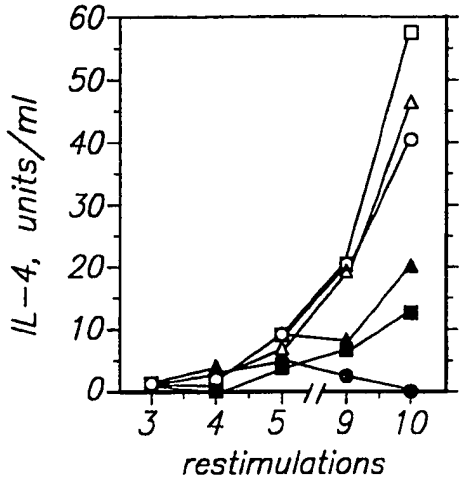


FIG. 2C

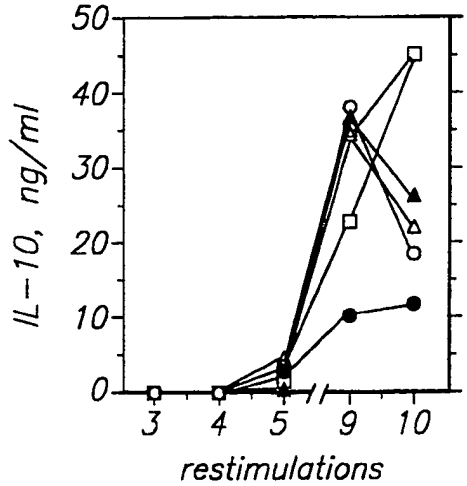


FIG. 2D

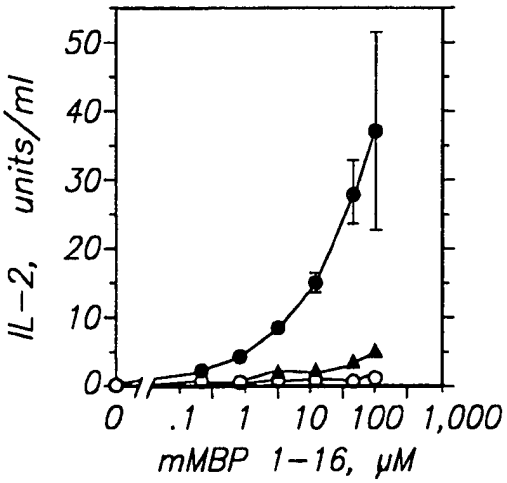
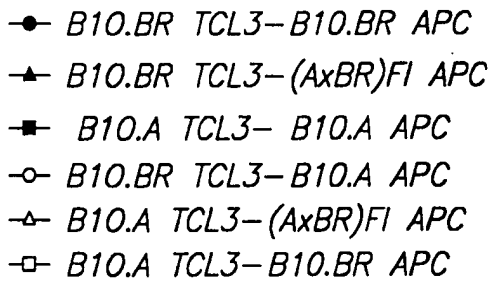


FIG. 2E



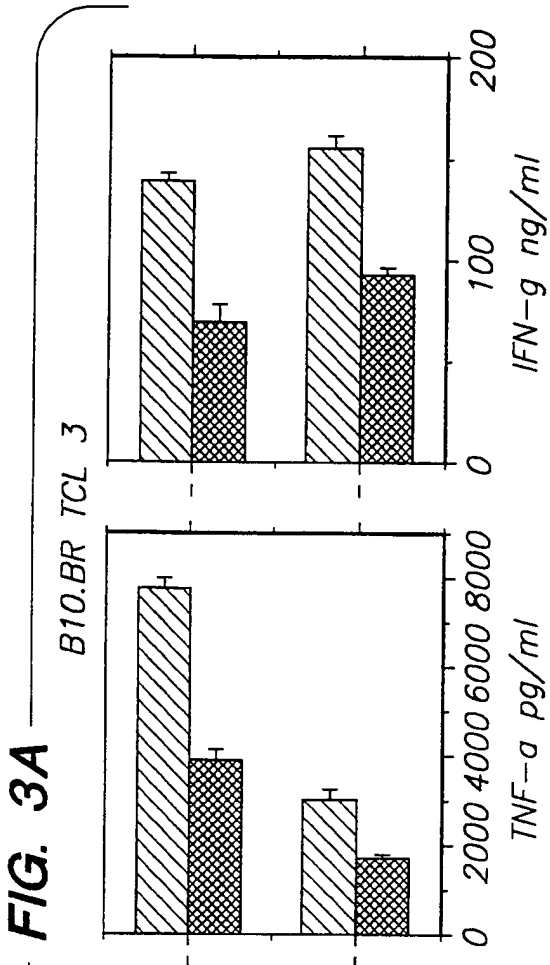


FIG. 3A
B10.BR TCL 3
Splenic APC
B10.BR
B10.A
B10.BR T-cell depleted
B10.A T-cell depleted
0 2000 4000 6000 8000
TNF-a pg/ml
0 100 200
IFN-g ng/ml

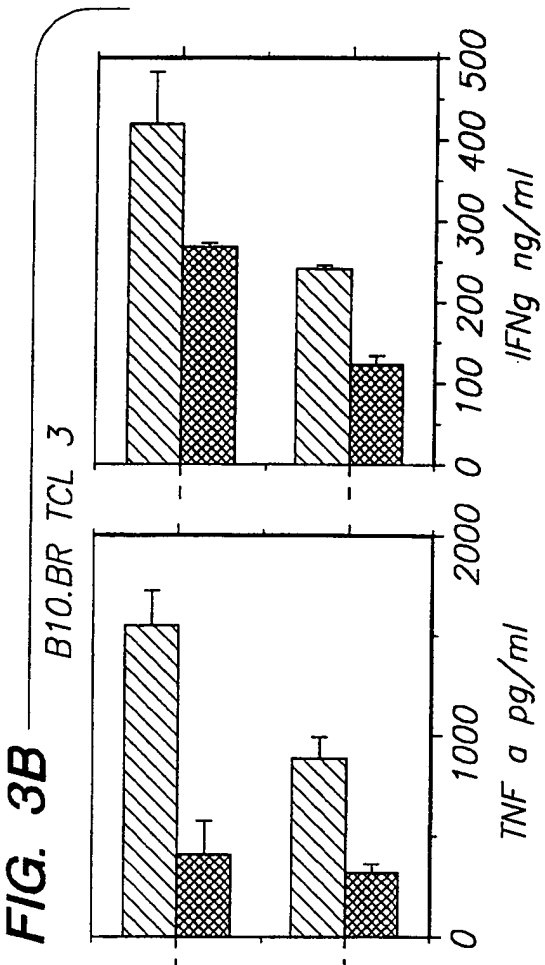


FIG. 3B
B10.BR TCL 3
APC 2hr sup
B10.BR
B10.A
B10.BR TCL+APC
B10.BR
B10.A TCL+APC
0 1000 2000
TNF a pg/ml
0 100 200 300 400 500
IFNg ng/ml

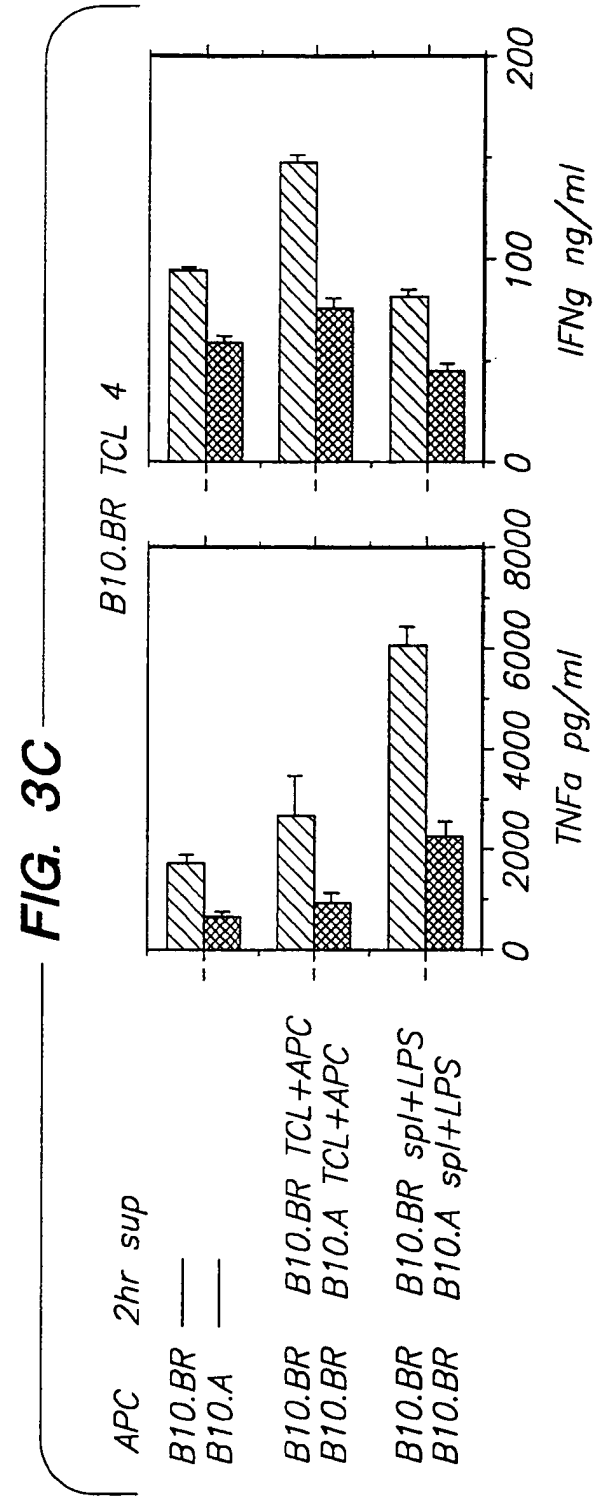

B10.BR TCL 4
0 2000 4000 6000 8000
TNFa pg/ml
0 100 200
IFNg ng/ml
APC 2hr sup
B10.BR
B10.A
B10.BR TCL+APC
B10.BR B10.A TCL+APC
B10.BR spl+LPS
B10.BR B10.A spl+LPS

FIG. 3C

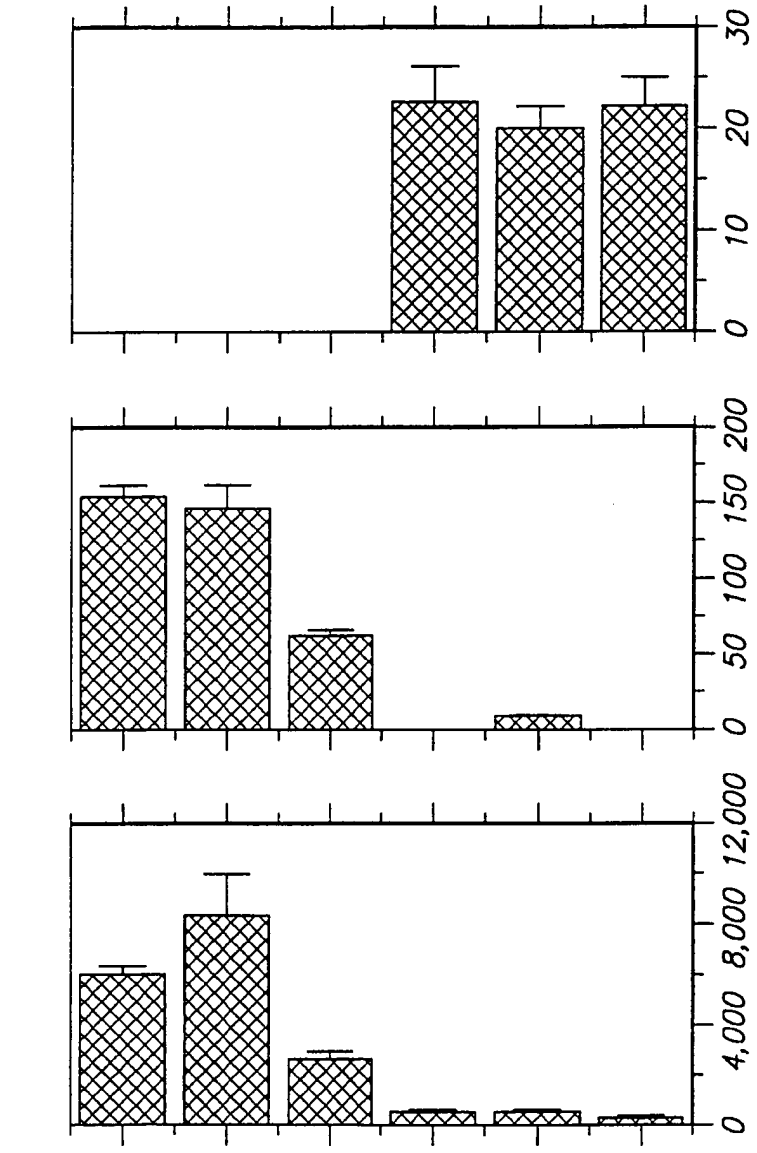


FIG. 4A

FIG. 4B

FIG. 4C

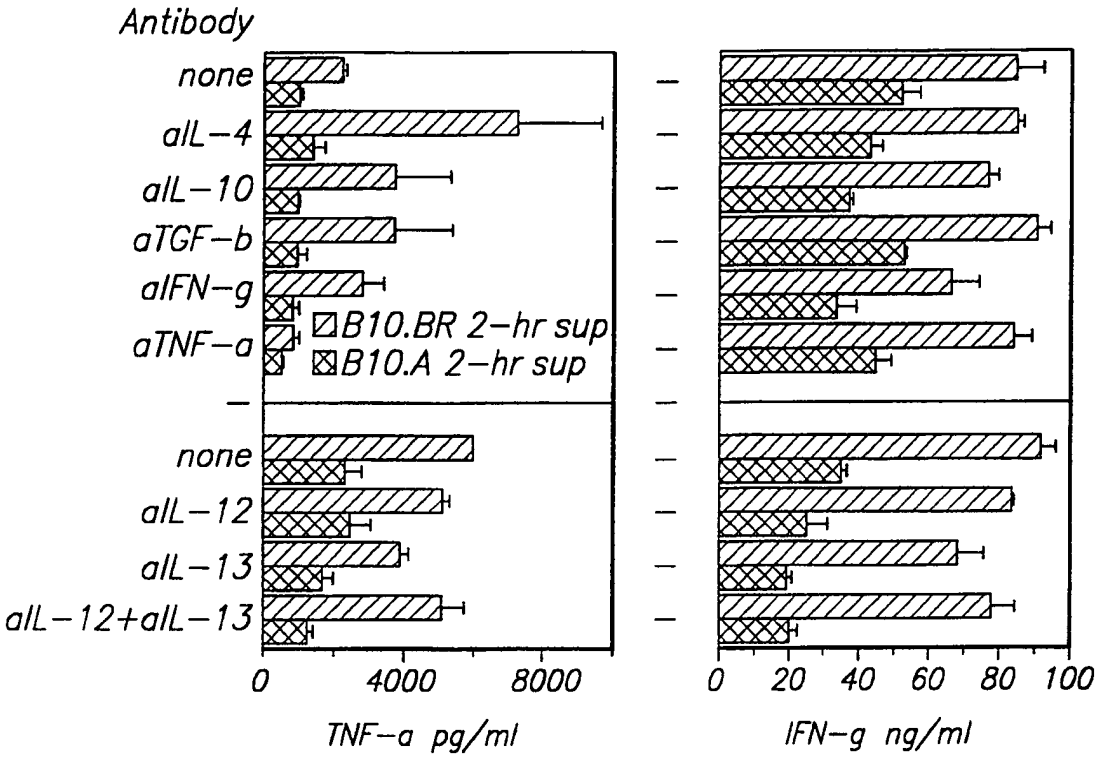


FIG. 5A

FIG. 5B

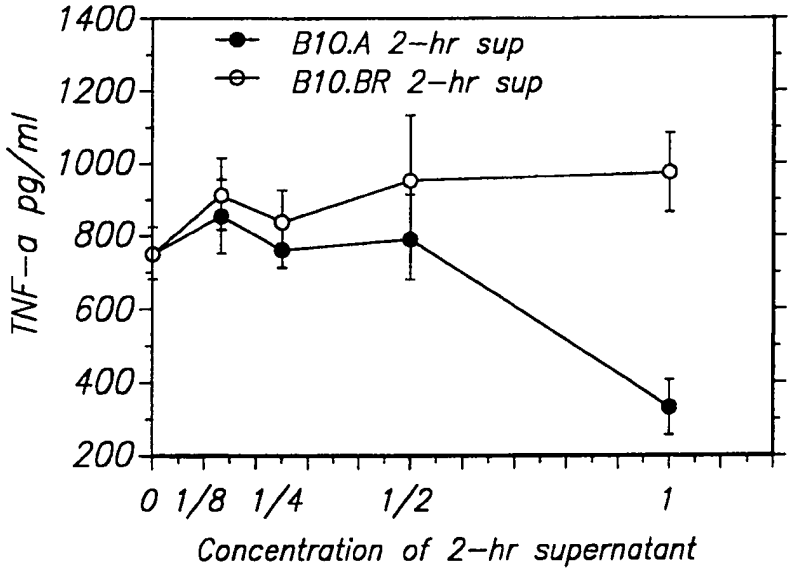


FIG. 5C

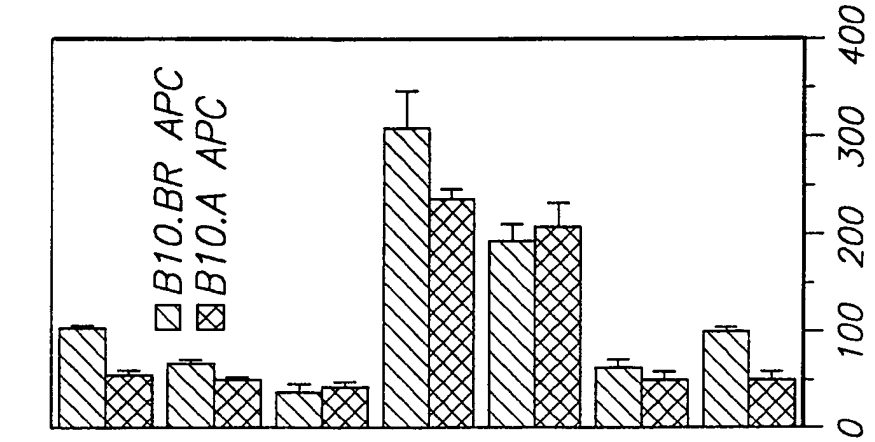

B10.BR APC
B10.A APC
0
100
200
300
400
IFN-γ % of control

FIG. 6B

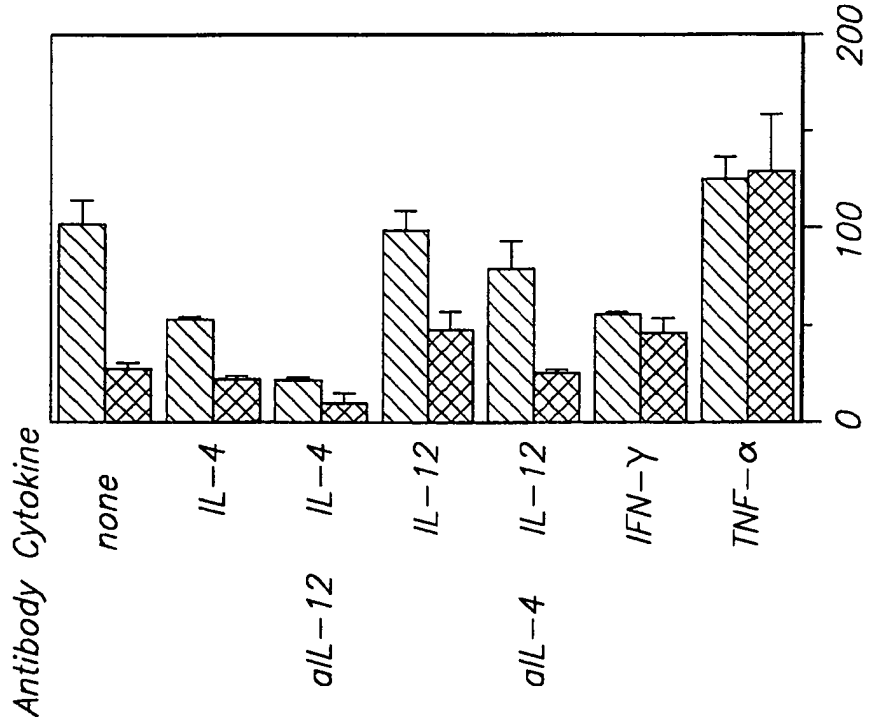

Antibody Cytokine
none
IL-4
αIL-12 IL-4
IL-12
αIL-4 IL-12
IFN-γ
TNF-α
0
100
200
TNF-α % of control

FIG. 6A

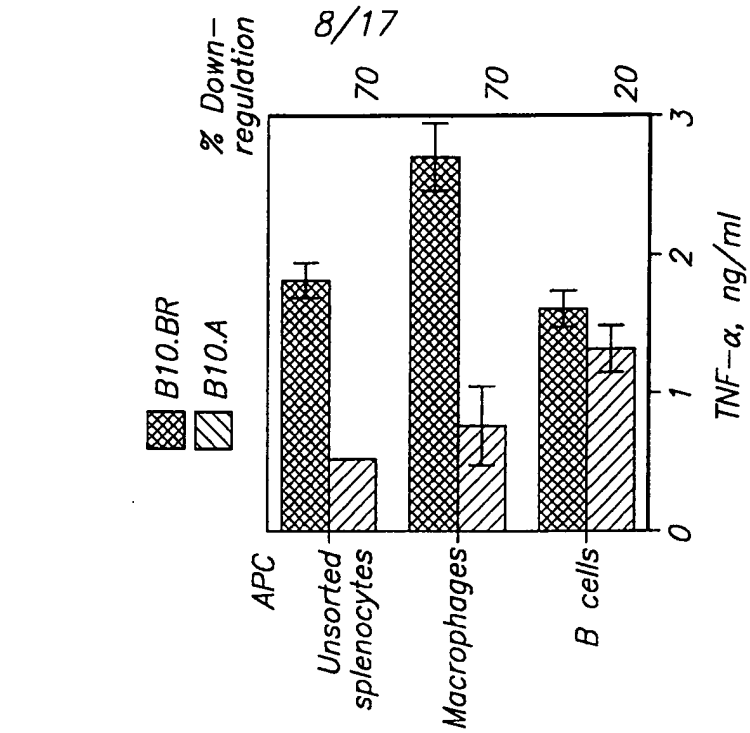


FIG. 8

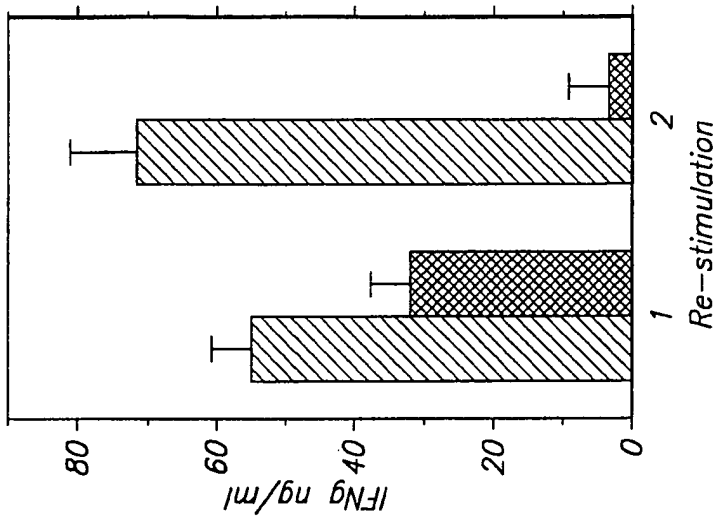


FIG. 7B

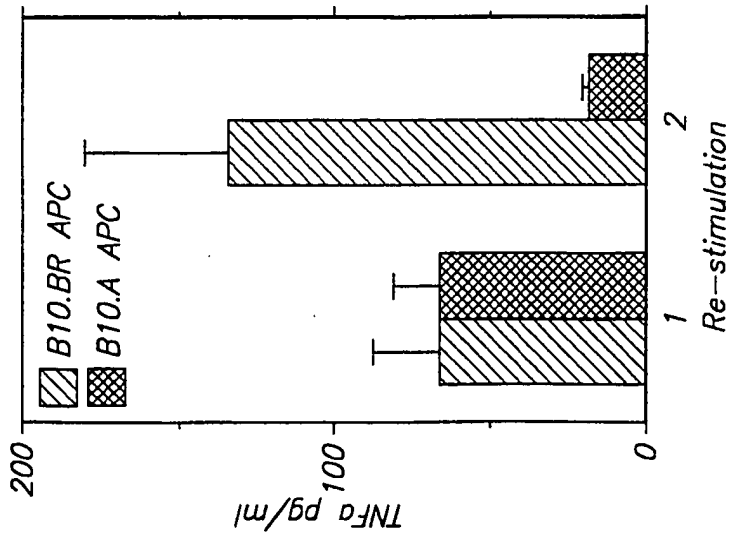


FIG. 7A

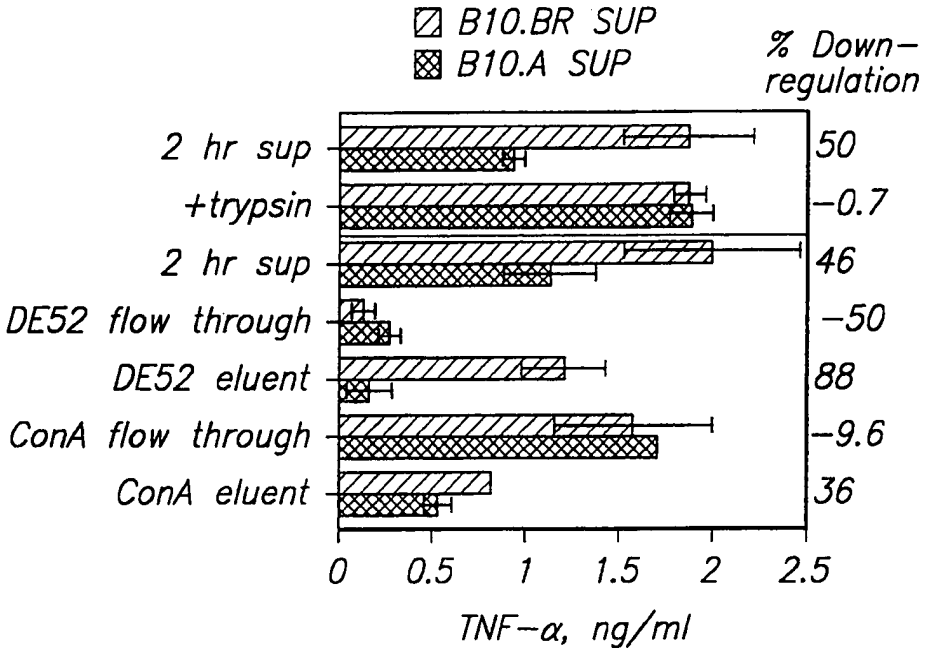

B10.BR SUP
B10.A SUP
% Down-regulation
2 hr sup
+trypsin
2 hr sup
DE52 flow through
DE52 eluent
ConA flow through
ConA eluent
50
-0.7
46
-50
88
-9.6
36
0
0.5
1
1.5
2
2.5
TNF-α, ng/ml

FIG. 9A

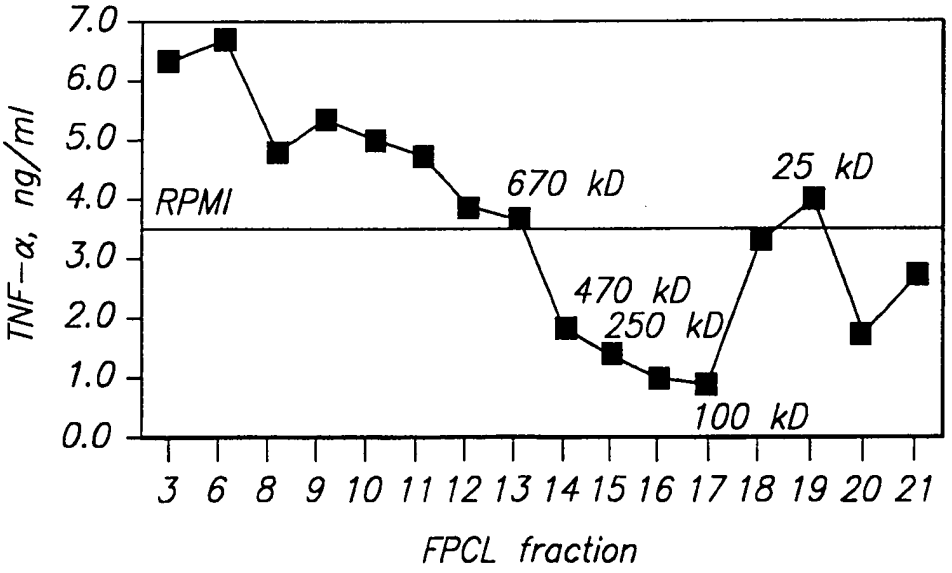

7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
TNF-α, ng/ml
RPMI
670 kD
25 kD
470 kD
250 kD
100 kD
3 6 8 9 10 11 12 13 14 15 16 17 18 19 20 21
FPCL fraction

FIG. 9B

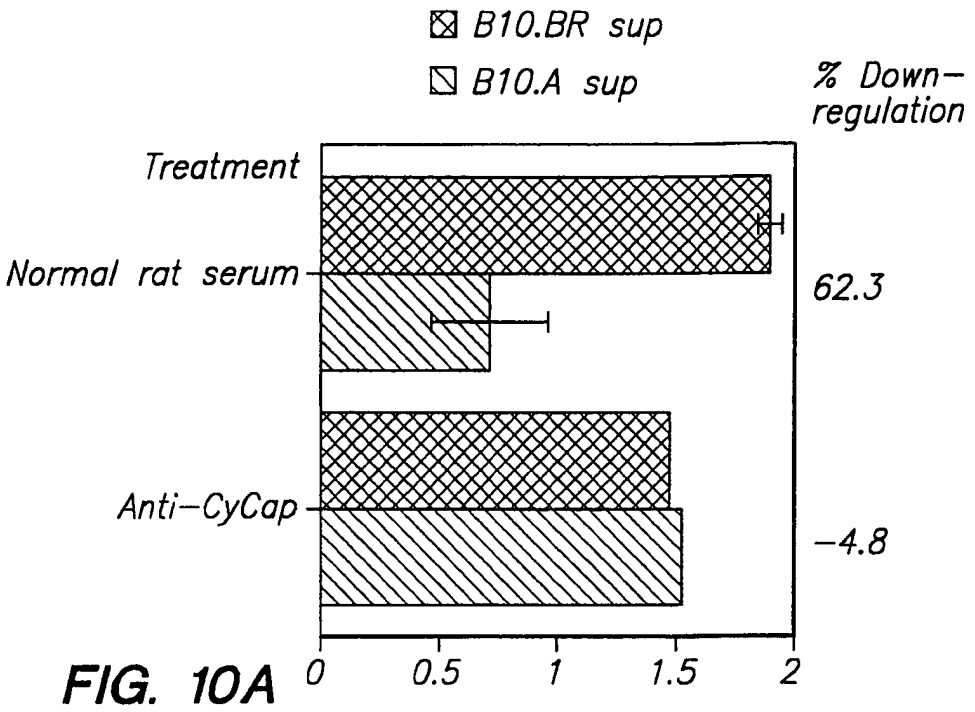


FIG. 10A

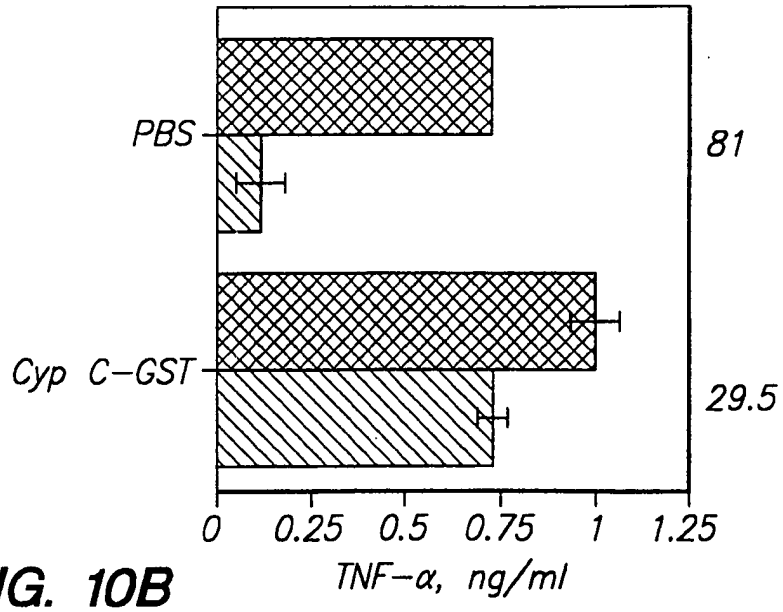


FIG. 10B

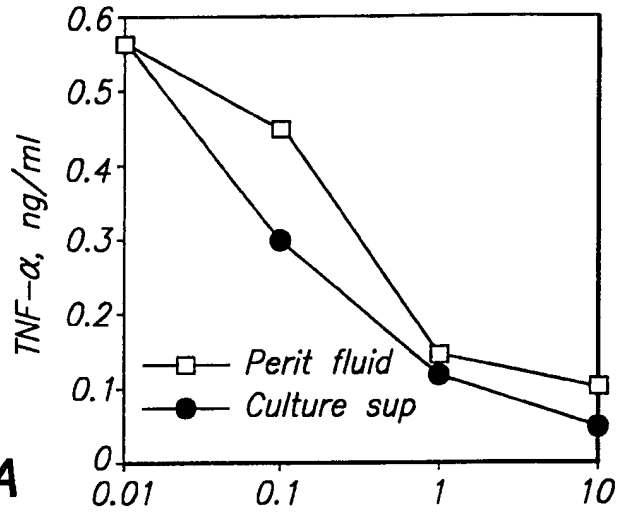


FIG. 11A

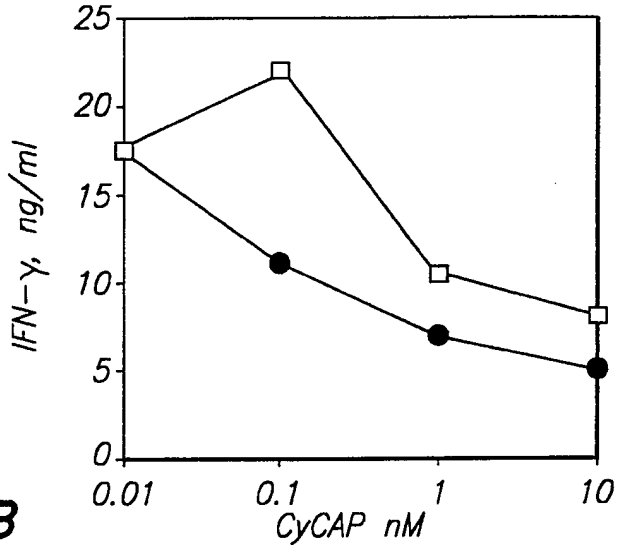


FIG. 11B

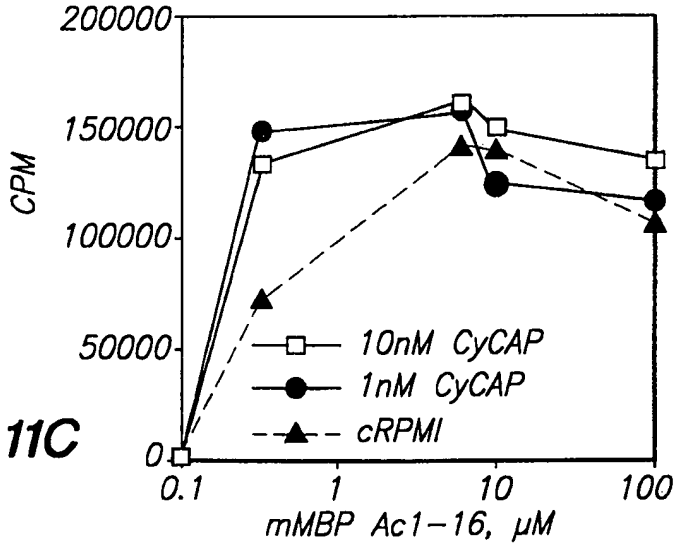


FIG. 11C

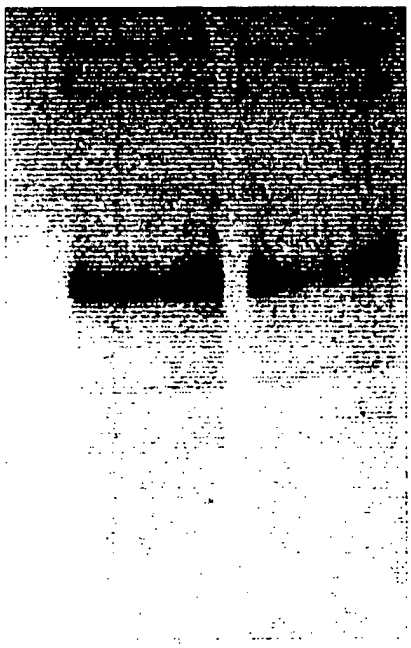


FIG. 11D

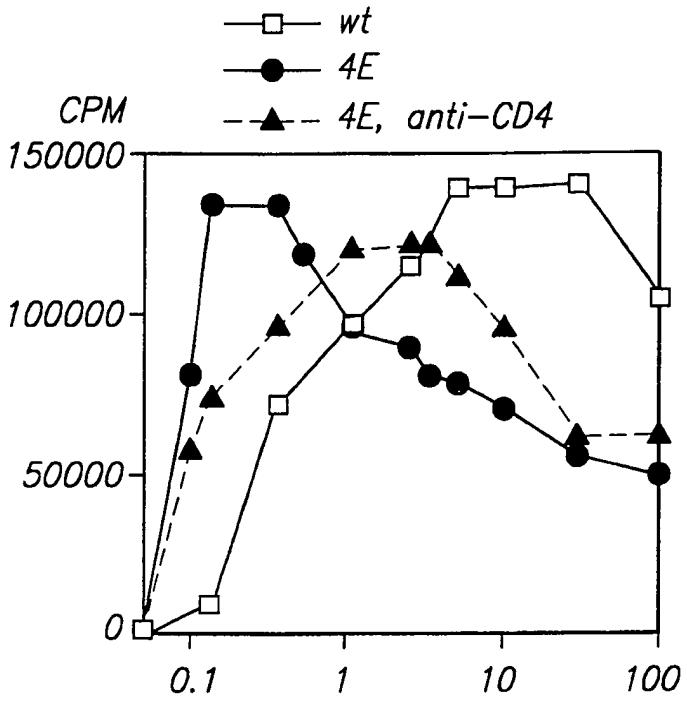


FIG. 12A

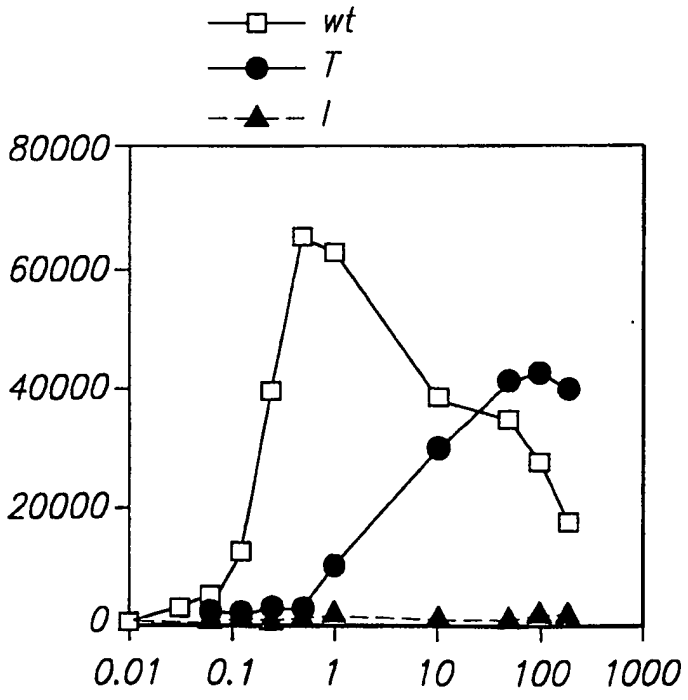


FIG. 12B

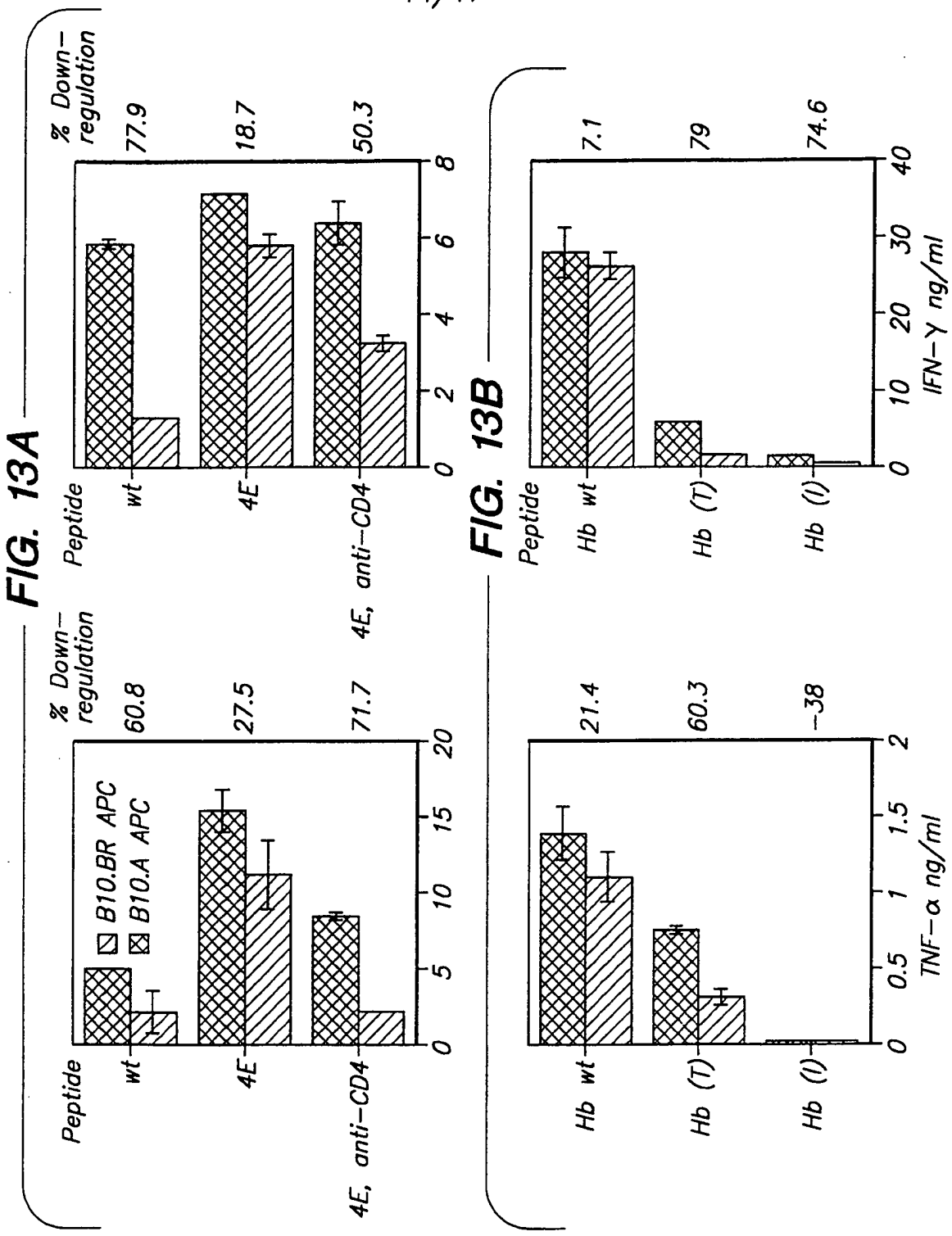


FIG. 13A
% Down-regulation
77.9
18.7
50.3
Peptide
wt
4E
4E, anti-CD4
0 2 4 6 8
% Down-regulation
60.8
27.5
71.7
B10.BR APC
B10.A APC
Peptide
wt
4E
4E, anti-CD4
0 5 10 15 20
FIG. 13B
7.1
79
74.6
Peptide
Hb wt
Hb (T)
Hb (I)
0 10 20 30 40
IFN-γ ng/ml
21.4
60.3
-38
Hb wt
Hb (T)
Hb (I)
0 0.5 1 1.5 2
TNF-α ng/ml

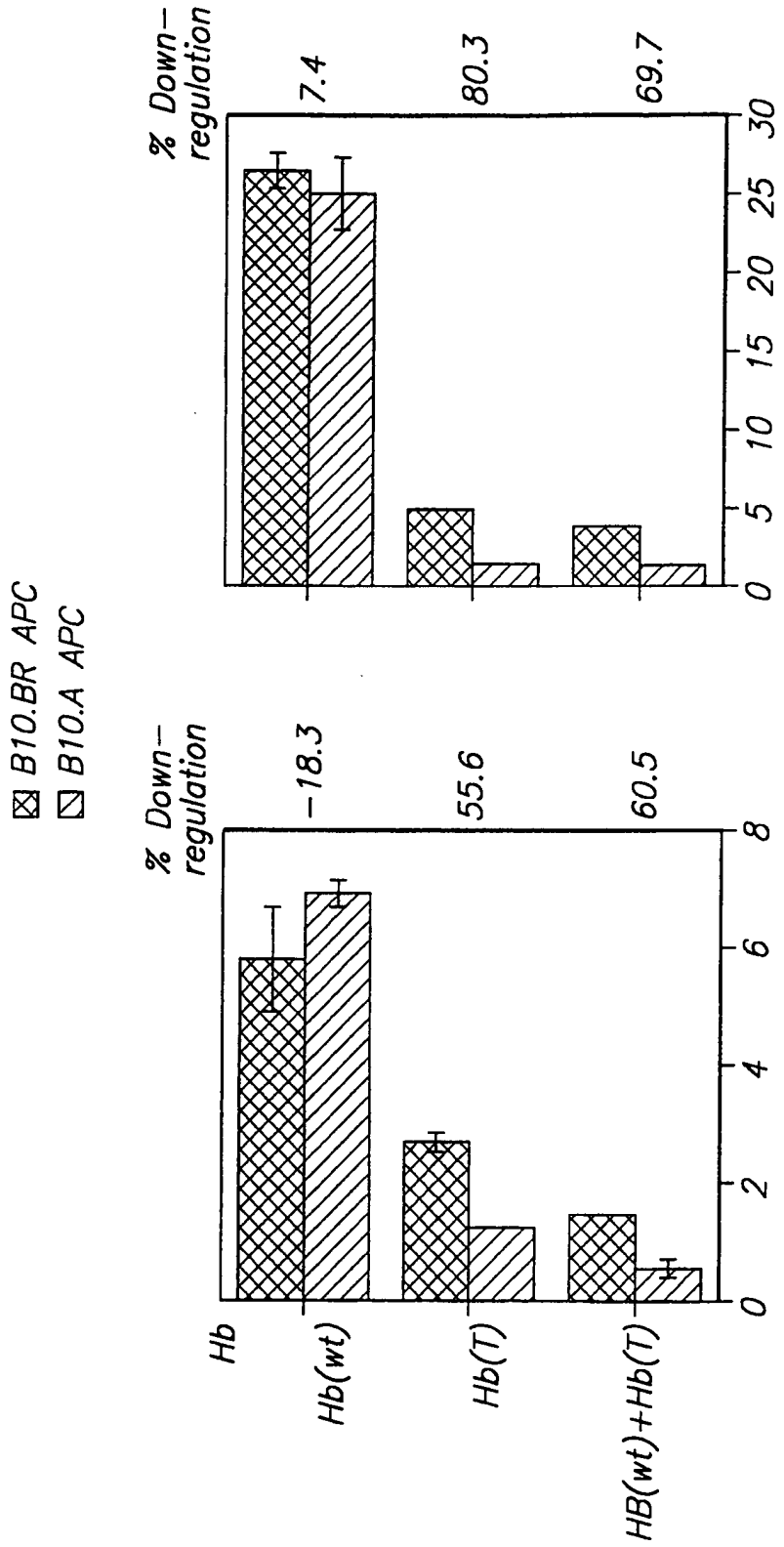


FIG. 14A

FIG. 14B

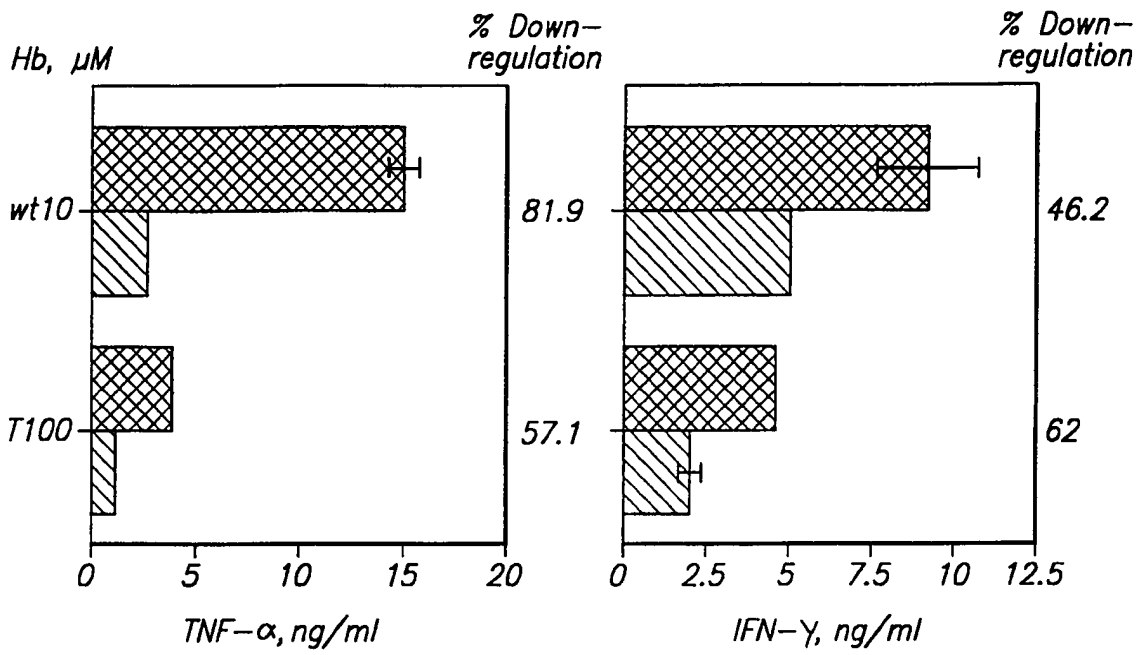


FIG. 15A

FIG. 15B

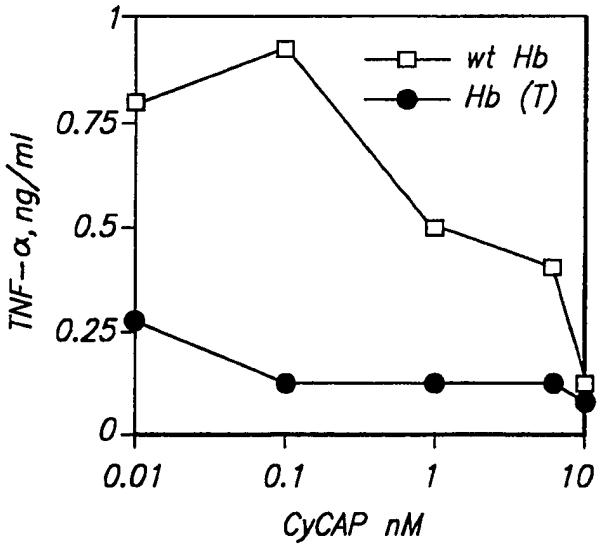


FIG. 15C

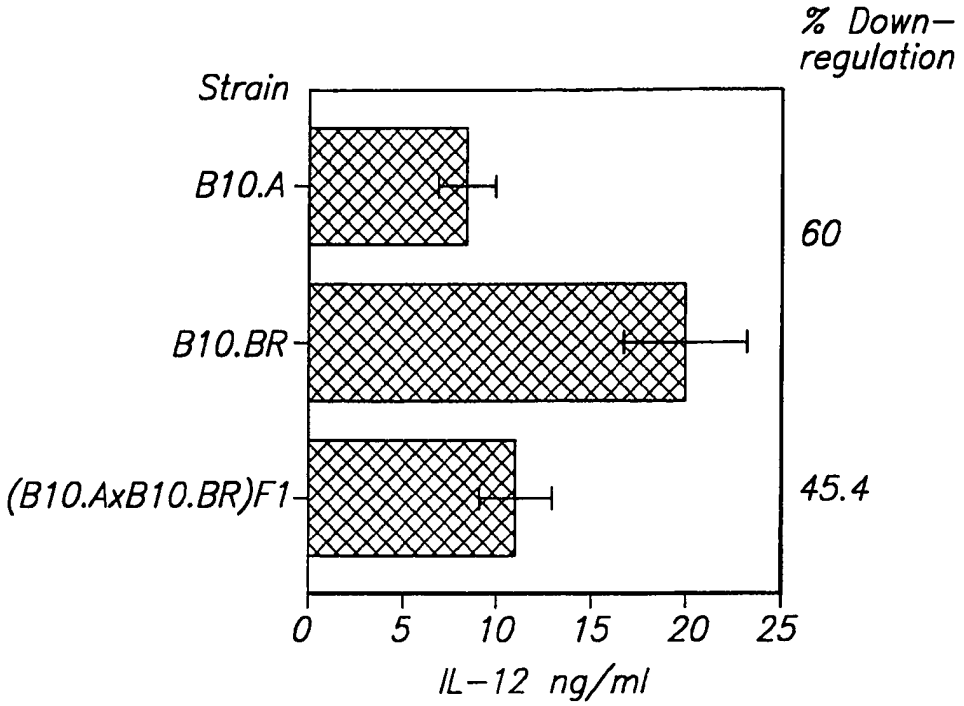


FIG. 16A

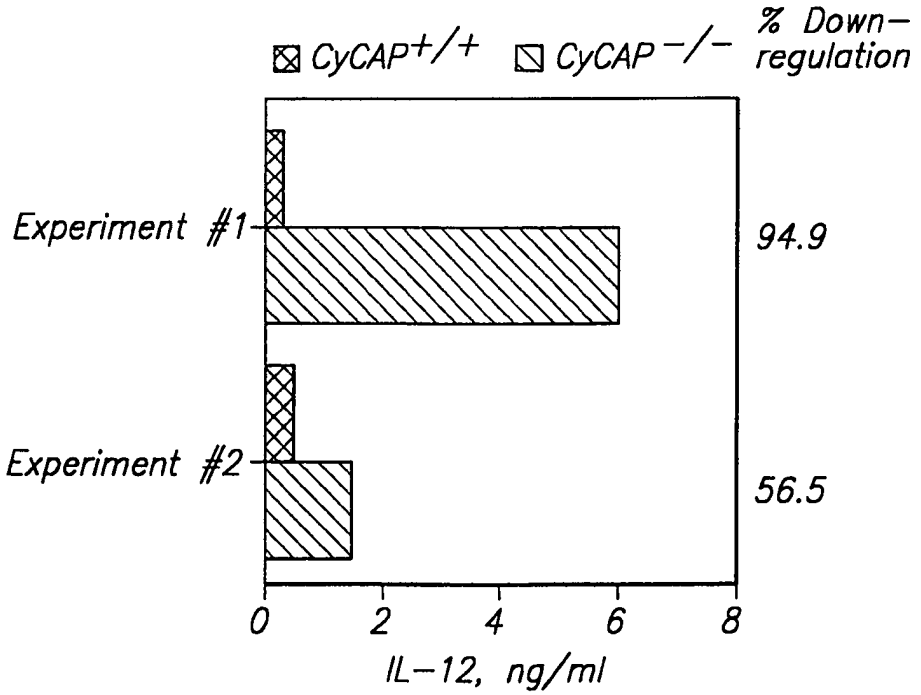


FIG. 16B

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/12345

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C07K 14/47; A61K 38/16, 38/17, 38/18
US CL :424/84; 514/2, 8, 12, 885; 530/350

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/84; 514/2, 8, 12, 885; 530/350

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

NONE

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	NATOLI et al. 90K Protein: A New Predictor Marker of Disease Progression in Human Immunodeficiency Virus Infection. Journal of Acquired Immune Deficiency Syndromes. 1993, Vol. 6, No. 4, pages 370-375.	1-11
A	KOTHS et al. Cloning and Characterization of a Human Mac-2-binding Protein, a New Member of the Superfamily Defined by the Macrophage Scavenger Receptor Cysteine-rich Domain. Journal of Biological Chemistry. 05 July 1993, Vol. 268, No. 19, pages 14245-14249.	1-11
A	IACOBELLI et al. Purification and characterization of a 90 kDa protein released from human tumors and tumor cell lines. FEBS Letters. March 1993, Vol. 319, No. 1,2, pages 59-65.	1-11

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"B"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search	Date of mailing of the international search report
19 AUGUST 1998	28 SEP 1998
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer PREMA MERTZ Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/12345

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	CHICHEPORTICHE et al. Cloning and Expression of a Mouse Macrophage cDNA Coding for a Membrane Glycoprotein of the Scavenger Receptor Cysteine-rich Domain Family. Journal of Biological Chemistry. 25 February 1994, Vol. 269, No. 8, pages 5512-5517.	1-11
X	ULLRICH et al. The Secreted Tumor-associated Antigen 90K Is a Potent Immune Stimulator. Journal of Biological Chemistry. 15 July 1994, Vol. 269, No. 28, pages 18401-18407, especially pages 18404 and 18406.	1, 3, 7-11

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/12345

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

APS, CAS ONLINE, MEDLINE, CAPLUS, BIOSIS
search terms: cyclophilin C-associated protein, cytokine regulatory factor, 90K protein, Mac-2 binding protein, murine adherent macrophage protein, administration, therapy, treatment.